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Factor Xa cleavage of fusion proteins. Elimination of non-specific cleavage by reversible acylation.

Wearne SJ.

MRC Laboratory of Molecular Biology, Cambridge, UK.

A method is described for the elimination of non-specific cleavage of fusion proteins by factor Xa. Putative non-specific cleavage sites C-terminal to lysyl residues are blocked by reversible acylation by 3,4,5,6-tetrahydrophthalic anhydride prior to cleavage. After cleavage, the acyl groups are removed quantitatively by exposure to slightly acidic conditions. This method employs no harsh reagents or conditions, and may be generally applicable to factor Xa cleavage of fusion proteins.

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Factor Xa cleavage of fusion proteins

Elimination of non-specific cleavage by reversible acylation

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Received 8 January 1990

A method is described for the elimination of non-specific cleavage of fusion proteins by factor Xa. Putative non-specific cleavage sites C-terminal to lysyl residues are blocked by reversible acylation by 3,4,5,6-tetrahydrophthalic anhydride prior to cleavage. After cleavage, the acyl groups are removed quantitatively by exposure to slightly acidic conditions. This method employs no harsh reagents or conditions, and may be generally applicable to factor Xa cleavage of fusion proteins.

Fusion protein cleavage; Factor Xa; Protein modification

1. INTRODUCTION

Efficient production of specific proteins by recombinant DNA techniques often requires expression of a fusion protein. To release the desired protein, the fusion protein must be cleaved at a specific site, which cannot also occur within any of the proteins to be made. Specific proteases can be used for this purpose.

The expression vector pLcIIFX created by Nagai and Thøgersen [1,2] contains, at the junction between the partial cII sequence and the site of the sequence of the protein to be expressed, a sequence encoding the tetrapeptide recognition sequence of blood coagulation factor Xa for prothrombin (Ile-Glu-Gly-Arg) [3]. Factor Xa cleaves the fusion protein (CIIFX-protein) at the C-terminal side of the tetrapeptide sequence, liberating authentic recombinant protein [1]. This expression system has been used for the production of several eukaryotic proteins in *E. coli* [2,4], including globins [5–7].

Factor Xa has been observed to cleave at additional sites other than the tetrapeptide recognition sequence in some fusion proteins [2,5], including wild type and mutant CIIFX-porcine myoglobin (pMb) used in this study. Non-specific cleavage of the pMb fusion protein is shown here to be due to factor Xa, to be likely to occur at lysyl residues, and to be inhibited by the reversible acylation of the fusion protein amino groups by THPA. Both acylation by THPA and deacylation may be achieved under gentle conditions [8,9]; this is therefore a method of potentially general use for the protection of fusion proteins from non-specific cleavage after lysyl residues by factor Xa.

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Abbreviations: THPA, 3,4,5,6-tetrahydrophthalic anhydride; pMb, porcine myoglobin; P37Y, 37Pr —→Tyr

2. EXPERIMENTAL

2.1. *Construction of pLcIIFX-porcine myoglobin mutants*

Porcine myoglobin was expressed from the M13mp18FXpmyo template [7]. Most of the studies were carried out on a mutant form (P37Y), in which the template was altered by site-directed mutagenesis [10,11]. Mutant clones were identified by sequencing along their entire length [12]. The 600-bp *Bam*HI/*Hin*dIII digest fragment was ligated into pLcII [2]. The ligation mixture was transfected into *E. coli* QY13; clones were selected by small-scale protein induction assay [2].

2.2. *Induction, expression and purification of mutant fusion protein*

Protein synthesis and inclusion body preparation were according to Nagai and Thøgersen [2]. The fusion protein was solubilized in urea-PED (8 M urea, 50 mM sodium phosphate (pH 8.0), 1 mM EDTA, 1 mM DTT), clarified by centrifugation and purified by gel filtration on a Sephacryl S-200 column pre-equilibrated with urea-PED. The concentrations of fusion protein samples were determined from their absorbance at 280 nm in 6 M guanidinium chloride, 20 mM sodium phosphate (pH 6.5), using extinction coefficients derived from the tryptophan and tyrosine content [13] (ϵ_{280} = 13.9 $mM^{-1} \cdot cm^{-1}$).

2.3. *THPA acylation of mutant fusion protein*

THPA acylation was achieved according to Gibbons and Schachman [8,9]. THPA (0.5 M in acetone) was added, with stirring, to the fusion protein sample at 4°C. If necessary, 0.5 M NaOH was added dropwise to maintain a pH of 8–8.5. After 30 min, the sample was dialyzed exhaustively against 50 mM Tris-HCl (pH 8.0), 0.5 M urea, 1 mM $CaCl_2$ at 4°C.

2.4. *Preparation of protease inhibitor-treated factor Xa*

Separate 15 μl fractions, each containing 9 μg factor Xa, were incubated with one of the following protease inhibitors prior to inclusion in a fusion protein cleavage reaction:

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(i) 5 U hirudin (type H7380, 500 U/mg, Sigma Chemical), at final reaction conditions f 50 mM Tris-HCl (pH 7.4), for 15 min at 37°C [14,15]. The degree of contaminant bdellin (trypsin-plasmin inhibitor) activity [16] in the hirudin preparation was determined by assaying the activity of trypsin solutions, according to Kassell [17], after preincubation with the hirudin preparation for 15 min at 37°C in 0.2 M triethanolamine (pH 7.8), 10 mM $CaCl_2$. Comparison with titration curves of trypsin inhibitor activity for pure bdellins [18] permitted estimation of the bdellin content of the hirudin preparation.

(ii) 12.5 nmol E-64 (Sigma Chemical), prepared in 10% DMSO, 0.1% Triton X-100, for 15 min at 25°C [19].

(iii) 5 U bovine anti-thrombin III (Sigma Chemical), at final reaction conditions of 0.1 M NaCl, 0.1 M Tris-HCl (pH 8.3), for 15 min at 37°C [20].

(iv) 30 μg BPTI (Bayer AG), at final reaction conditions of 67 mM sodium phosphate (pH 8.0), for 15 min at 25°C [21,22].

2.5. *Cleavage of fusion protein with factor Xa*

Non-THPA acylated CIIFX-pMb fusion proteins are only partially soluble at 0.45 mg/ml after dialysis against 50 mM Tris-HCl (pH 8.0), 0.5 M urea, 1 mM $CaCl_2$, whereas THPA acylated fusion proteins are fully soluble after dialysis. Cleavage with factor Xa was performed at 28°C at an enzyme to substrate ratio of 1:80 (w/w).

2.6. *Removal of THP groups*

Dialysis for 24–40 h at 4°C and pH 6 is a general method for the quantitative removal of THP groups from modified proteins [8,9]. pMb precipitates under these conditions, however, as this pH is close to the p*I* of the protein, and quantitative deacylation was not observed. Quantitative removal of THP groups from pMb can be produced using conditions where the protein remains soluble, such as dialysis against 0.1 M acetic acid for 20 h, or addition of guanidinium chloride to 5 M and incubation at 4°C for 20 h.

2.7. *Purification of porcine myoglobin*

If the removal of acylating groups by dialysis was not quantitative, the deacylated species was purified by anion exchange chromatography on a DEAE-Sepharose CL6B column (40 cm × 1 cm diameter), equilibrated with 4.5 M urea, 25 mM Tris-HCl (pH 7.6). The sample was loaded in the same buffer, and eluted with a 200 ml linear gradient of 0–0.1 M NaCl.

2.8. *Electrophoretic analysis*

Samples fr m cleavage reactions were analyzed by SDS-polyacrylamide gel electr phoresis, according to Schaegger and Von Jag w [23]. A 16.5% T, 3% C separating gel was used in all instances, with a 10% T, 3% C spacer gel.

The degree of THPA acylation of samples was determined by polyacrylamide gel electrophoresis [24], with 8 M urea included in gel mixtures and samples [8].

3. RESULTS AND DISCUSSION

Cleavage of unmodified CIIFX-pMb (P37Y) with factor Xa generates several protein species by non-specific cleavage, in addition to authentic myogl bin (Fig. 1a). The rate of non-specific cleavage is not affected significantly by E-64 (Fig. 1b), a specific inhibitor of cysteine proteases, or by hirudin (Fig. 1c), a specific inhibitor of thrombin. The hirudin preparation was found to contain bdellins at 25–30% by weight (a level comparable to those reported for other crude preparations [16]), therefore non-specific cleavage is not due to plasmin activity. However, both anti-thrombin III (a specific inhibitor of thrombin and factor Xa), and BPTI (a general inhibitor of serine proteases) inhibit all cleavage (Fig. 1d, e). It is likely, therefore, that non-specific cleavage is due to factor Xa, not to proteases present as contaminants.

The degree of non-specific cleavage varies between wild type and mutant myoglobin species, with mutant CIIFX-pMb (P37Y) the most susceptible. This implies a dependence of cleavage not only on primary sequence, but also on conformation and accessibility. The molecular weights of the non-specific cleavage products were determined by SDS-polyacrylamide gel elec-

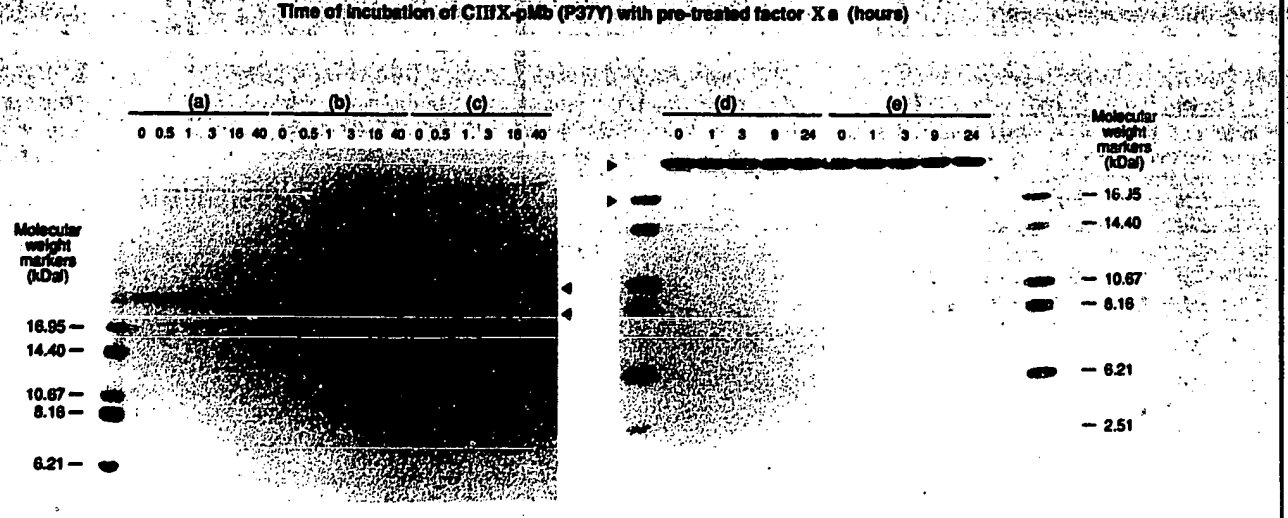

Fig. 1. SDS-PAGE f species generated by cleavage of unmodified CIIFX-pMb (P37Y) by protease inhibitor-treated factor Xa. The inhibitors used, and their concentration per 9 μg aliquot of factor Xa were: (a) no treatment; (b) 12.5 nmol E-64; (c) 0.5 U hirudin; (d) 0.5 U anti-thrombin III; (e) 30 μg BPTI. The molecular weights of cleavage products are given in Table I. Treatments a, b, c and treatments d, e were run on different gels; in each case, the upper arrowhead identifies the fusion protein (approx. mol. wt. 21200 kDa) and the lower arr w identifies pMb (P37Y) (approx. mol. wt. 17400 kDa).

Table I

Major cleavage products generated on digesti n f unmodified CIIFX-pMb (P37Y) by factor Xa

Polypeptide molecular weight[a]	Putative cleavage site at the peptide bond C-terminal to:
21380 ± 200[b]	None
20230 ± 320	Lys145 (or Arg139)
18790 ± 310	Lys133
17700 ± 290[c]	FXa cleavage site
15740 ± 290	FXa cleavage site and Lys145 (or Arg139)
15170 ± 70	Lys96 or Lys98

[a] Error limits are the standard deviation in molecular weight ($n = 5$)
[b] CIIFX-pMb (P37Y) fusion protein
[c] Authentic pMb (P37Y)

trophoresis. Putative cleavage sites were identified from the calculated molecular weights (Table I), which suggested that cleavage was occurring at lysyl residues.

The primary sequences of pMb [25,26] and β-globin [27,28] were compared to identify a possible basis for the non-specific cleavage that occurs with the former, but not the latter. There is a significant difference between β-globin and pMb in the number of lysyl residues they contain (11 and 19, respectively), and in their distribution throughout the protein. In pMb, 4 regions may be identified in which clustering of lysyl residues occurs, giving sequences with multiple positive charge. Only one such region occurs in β-globin, suggesting that factor Xa was cleaving at sites of high positive charge.

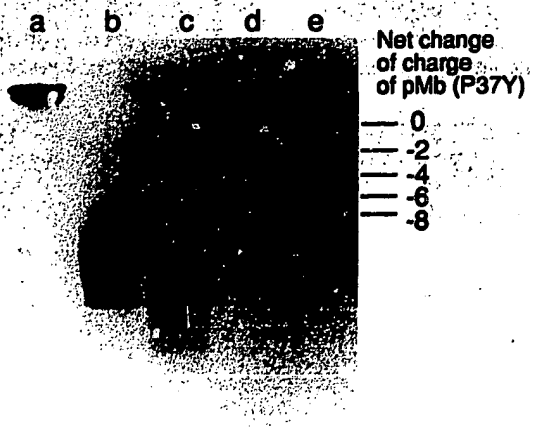

Fig. 2. Comparison of the degree of deacylation achieved by dialysis against 0.1 M acetic acid and dialysis against 50 mM citrate buffer (pH 5.8), by urea-PAGE of dialysates: (a) unmodified fusi n protein CIIFX-pMb (P37Y); (b) THP acylated fusion protein; (c) acylated pMb (P37Y) generated by factor Xa cleavage; (d) acylated pMb (P37Y) after dialysis against 50 mM citrate buffer (pH 5.8) for 20 h at 4°C; (e) acylated pMb (P37Y) after dialysis against 0.1 M acetic acid for 20 h at 4°C.

The possibility that n n-specific cleavage was occurring at lysyl residues led t attempts to minimize it by reversible acylation f the fusion protein. The most readily reversible acylati n reagent is THPA [8]. Acylati n f the CIIFX-pMb species with THPA leads to a change of net charge of −2 for each amino group acylated [8]. Electrophoretic analysis of acylated samples demonstrated primarily the expected species, but also some with changes of net charge of −3, −5, etc. The presence of fusion protein with a change of net charge of −1, prior to blocking (Fig. 2a), indicates that another covalent modification, such as spontaneous deamidation [29] or carbamylation by cyanate on the degradation of urea [30], occurs in addition to any possible acylation by contaminants of the commercial THPA preparation and is at least partly responsible for the generation of these species. The minor species are separated from unmodified pMb by anion exchange chromatography.

A stoichiometry of 0.8 THPA molecules per free amino group was empirically determined to be sufficient to eliminate non-specific cleavage (Fig. 3). The observation that THP acylation with a stoichiometry of less than 1 is sufficient to suppress cleavage is unlikely to be applicable to all proteins. Native pMb contains 4 clusters of lysyl residues; it is possible that either THP acylation of all lysyl α-amines in such clusters is sterically unfavored, or that partial acylation of each cluster is sufficient to eliminate potential cleavage within that region.

Generally, THP groups can be removed very easily, without the need for harsh reagents or conditions [8,9], which makes this method preferable to trifluoroacetylation [31] or acylation by citraconic anhydride [32] for masking amino groups. However, dialysis at pH 5.8–6.0 leads to precipitation of pMb and pMb (P37Y), and non-denaturing polyacrylamide gel electrophoresis of the dialysate in the presence of

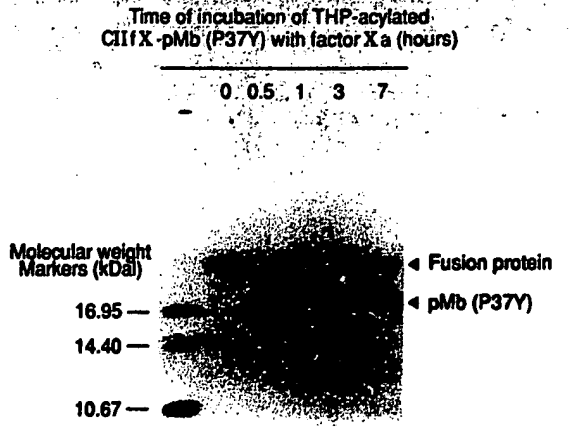

Fig. 3. SDS-PAGE of the products of cleavage f THP acylated CIIFX-pMb (P37Y) (0.8 THPA molecules per free amin group) by factor Xa.

urea shows that deacylati n is not quantitative (Fig. 2d). Quantitative deacylati n f the pMb species theref re requires different conditions from th se generally applicable t other pr teins, and was achieved by either dialyzing against 0.1 M acetic acid, r by incubating in 5 M guanidinium chloride at 4°C and pH 5.8–6.0 (Fig. 2e). After removal of minor charged species by anion exchange chromatography, the yield of pMb (P37Y) from deblocking by either acetic acid or guanidinium chloride incubation is typically 0.70 mg/g packed cells (wet weight). This compares favorably with 0.18 mg/g yielded by partial cleavage of non-acylated CIIFX-pMb (P37Y) (Fig. 1a), if fully quantitative separation of cleavage products is achieved.

Acylation by THPA may be suitable as a general method for eliminating non-specific cleavage of CIIFX-fusion proteins by factor Xa. Where the p*I* of the fusion protein is less than 8 (the pH at which factor Xa cleavage is routinely executed), the change in net charge on acylation by THPA may also increase the solubility of the fusion protein, accelerating the rate of cleavage.

Acknowledgements: Double-stranded M13mp11FXpmyo was kindly provided by Drs G. Dodson and S.J. Smerdon (University of York); expression vector pLcII was kindly provided by Dr K. Nagai (MRC Laboratory of Molecular Biology); factor Xa was kindly provided by Dr P. Esnouf (University of Oxford). Thanks are due to Dr T.E. Creighton for helpful discussions and for many useful comments in the preparation of this manuscript.

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A high throughput assay for inhibitors of HIV-1 protease. Screening of microbial metabolites.

Sarubbi E, Nolli ML, Andronico F, Stella S, Saddler G, Selva E, Siccardi A, Denaro M.

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A novel method for discovery of HIV-1 protease inhibitors in complex biological samples has been developed. The assay is based on two specific reagents: a recombinant protein constituted by a portion of the HIV-1 Gag polyprotein comprising the p17-p24 cleavage site, fused to E. coli beta-galactosidase, and a monoclonal antibody which binds the fusion protein in the Gag region. Binding occurs only if the fusion protein has not been cleaved by the HIV-1 protease. The assay has been adapted for the screening of large numbers of samples in standard 96-well microtiter plates. Using this method about 12000 microbial fermentation broths have been tested and several HIV-1 protease inhibitory activities have been detected. One of these has been studied in detail.

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A high throughput assay for inhibitors of HIV-1 protease

Screening of microbial metabolites

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Received 13 December 1990

A n vel method for discovery of HIV-1 protease inhibitors in complex biological samples has been developed. The assay is based on two specific reagents: a recombinant protein constituted by a portion of the HIV-1 Gag polyprotein comprising the p17–p24 cleavage site, fused to *E. coli* β-galactosidase, and a monoclonal antibody which binds the fusion protein in the Gag region. Binding occurs only if the fusion protein has not been cleaved by the HIV-1 protease. The assay has been adapted for the screening of large numbers of samples in standard 96-well microtiter plates. Using this method about 12000 microbial fermentation broths have been tested and several HIV-1 protease inhibitory activities have been detected. One of these has been studied in detail.

Human immunodeficiency virus; Proteinase; Fusion protein; Immunological assay; Microbial alkaline protease inhibitor

1. INTRODUCTION

There is growing interest in developing specific inhibitors of the HIV-1 aspartyl protease as possible therapeutic agents in the treatment of AIDS [1,2]. Several different HIV-1 protease assays, based either on HPLC separations [3–7] or on chromophoric [8,9], fluorogenic [10–12] or radiolabeled [13–15] synthetic peptides have been developed in various laboratories to test a variety of compounds for inhibitory activity.

The 3D structure of the HIV-1 protease has recently been determined [16–18] and many investigators have made extensive use of the rational approach to design substrate analogs with specific inhibitory activity [19–23]. This has led to peptide derivatives which are potent inhibitors, but often suffer major drawbacks, e.g. scarce solubility in aqueous solutions, poor cell permeability, very rapid in vivo degradation, all factors causing serious limitations in bio-availability. As a consequence, there is at the present time a renewed interest in the screening of natural products for novel, possibly non-peptidic molecules which could display comparable inhibitory activity against HIV-1 protease and, at the same time, more interesting in vivo properties. In addition, in a random screening it is possible to discover inhibitors of dimer formation, a process which appears to be required for full HIV-1 protease activity [16–19,24].

With this in mind, we have used a biotechnology approach to develop an assay which is particularly suitable for the detection of HIV-1 protease inhibitors in large numbers of samples. The assay has been used to screen microbial fermentation broths and several inhibitory activities have been found.

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Abbreviations: HIV-1, human immunodeficiency virus type-1; HPLC, high performance liquid chromatography; IPTG, isopropyl-βD-thi galactoside; PMSF, phenylmethylsulphonyl fluoride; β-ME, β-mercaptoethanol; TPEG, *p*-amin phenyl-βD-thiogalactoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethane-sulphonic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PNPG, *p*-nitrophenyl-βD-galactoside; MAb, m noclonal antibody; α-MAPI, α-microbial alkaline protease inhibitor.

2. MATERIALS AND METHODS

2.1. *HIV-1 protease*

Recombinant *E. coli* expressing HIV-1 protease was kindly provided by Dr B. Harris [24]. For screening of fermentation broths the enzyme was routinely used as a crude preparation [24]. Briefly, after induction and harvesting, cells (50 g) were resuspended in 100 ml of 10 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 0.05% Triton X-100, sonicated and centrifuged at 10 000 × *g* for 20 min. HIV-1 protease activity in *E. coli* extracts was assayed by cleavage of a synthetic heptapeptide substrate and HPLC analysis of products (C. Tarnus, MMRDI, Strasbourg, personal communication), essentially as described [3]. A corresponding extract from *E. coli* cells lacking the HIV-1 protease expression vector was employed for controls.

2.2. *Expression and purification of gal-gag110*

The *Hin*dIII-*Hin*dIII fragment from bp 631 to 1258 f HIV-1 *gag* DNA [25] was subcloned int the unique *Hin*dIII site f pUC19 (Gibco BRL). From the resulting c nstruct the *Pst*I-*Pst*I fragment containing 8 bp from the pUC19 polylinker (*Pst*I-*Hin*dIII) fused to the *Hin*dIII-*Pst*I fragment from bp 631 to 961 f HIV-1 DNA, was

isolated and inserted into the unique *PstI* site of pUR292 [26]. This gave plasmid pGA22 in which the *gag* fragment is fused in frame t the end f the *lacZ* gene to encode a fusion protein. The structure of this 'gal-gag110' fusion protein is depicted in Fig. 1.

E. coli JM109(pGA22) was gr wn at 37°C in Luria Broth in the presence of 50 μg/ml ampicillin. At OD_{600} = 0.5 IPTG (1 mM) was added and after 2 h bacteria were harvested and resuspended in 1/50 volume of 50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM EDTA (Buffer A), containing 0.1 mM PMSF, 1 mM benzamidine-HCl, 0.1 M arginine and 1 mg/ml lysozyme. After 30 min at 4°C cells were disrupted by sonication and centrifuged for 10 min at 25 000 × *g*. The pellet was washed twice with Buffer A containing 70 mM β-mercaptoethanol (β-ME) and 0.05% Triton X-100 and then resuspended in 0.1 M Tris-HCl, pH 7.8, 0.1 M NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 70 mM β-ME (Buffer B), containing 8 M urea. After centrifugation (20 min at 50 000 × *g*), the supernatant was adjusted to 5 M urea by dilution with Buffer B, then extensively dialyzed against Buffer B without β-ME. Affinity chromatography on TPEG-Sepharose was performed essentially as described [27]. Protein purification was checked by SDS-PAGE using the PHAST-GEL system (Pharmacia, Sweden).

2.3. *Production of MAb 1G12*

BALB/c mice were immunized i.p. with 50 μg of gal-gag110 at days −60, −42, −30 and −18. At day −3 mice were boosted i.v. with the same protein. Spleen cells were fused with Sp2/0-Ag14 mouse myeloma cells and hybridoma supernatants were screened by ELISA techniques for antibodies against gal-gag110 which did not bind β-galactosidase. Clone 1G12 was isolated and subcloned by limiting dilution technique. MAb 1G12 was identified as IgG_1 using the Mouse-Typer Sub-isotyping Panel (Bio-Rad). A western Blot analysis confirmed that MAb 1G12 binds gal-gag110 with no detectable cross-reactivity with β-galactosidase.

2.4. *Immunoassay for detection of HIV-1 protease inhibitors*

After removal of microbial cells by centrifugation, 5 μl of fermentation broth were mixed in 96-well microtiter plates with 45 μl of 0.25 M MES/NaOH buffer, pH 6.0, 0.1 M NaCl, 0.6% w/v BSA, 0.025% v/v Tween 20, 1 mM sodium EDTA, 5 μg/ml leupeptin, 1 mM PMSF and 1% v/v *E. coli* extract containing recombinant HIV-1 protease (section 2.1). After 10 min of pre-incubation at room temperature to allow binding of a putative inhibitor to the viral enzyme, 25 μl of gal-gag110 fusion protein (50 μg/ml) were added to the wells and the microtiter plates incubated at 37°C for 40 min. 50 μl of reaction mixture were then transferred from each well into the corresponding well of a flat-bottom microtiter plate pre-coated with MAb 1G12.

These plates were coated by incubating 50 μl of MAb 1G12 (50 μg/ml in PBS) per well overnight at 25°C. Unreacted protein binding sites were blocked by incubation with 3% BSA in PBS (1 h, 25°C). Finally, wells were emptied, washed 4–5 times with PBS and pat-dried on paper towels. MAb 1G12-coated plates were usually used freshly made.

After addition of the reaction solutions, the MAb 1G12-coated plates were incubated for 2 h at 25°C to allow the selective binding of the uncleaved gal-gag110 to the antibody. The solutions were then discarded and the plates washed 4–5 times with PBS containing 0.05% Tween 20. To quantitate the amount of uncleaved gal-gag110, 240 μl/well of 1 mg/ml PNPG, a β-galactosidase-specific chromogenic substrate, in 50 mM sodium phosphate buffer, pH 7.8, 50 mM NaCl, 1 mM $MgCl_2$ and 70 mM β-ME were added and the plates incubated for 1 h at 25°C. Finally, 60 μl/well of 1.5 M Na_2CO_3 were added to stop the β-galactosidase reaction and the absorbance at 405 nm was determined using a Titertek Multiscan micr plate reader (Flow Labs).

3. RESULTS

3.1. *Production and characterization of gal-gag110*

In order to obtain an HIV-1 protease substrate which was specific and, at the same time, suitable for a simple solid-phase assay, a gene coding for a fusion pr tein was constructed. The resulting protein was engineered to contain the Ala^{100} to Ala^{210} fragment from HIV-1 Gag polyprotein (p55) fused to the carboxy-terminus of *E. coli* β-galactosidase (Fig. 1). The gag portion of this 'gal-gag110' fusion protein comprised the p17/p24 HIV-1 protease cleavage site Tyr^{132}-Pro^{133} [25], while the β-galactosidase portion provided an easily measurable enzymatic activity.

E. coli JM109 containing plasmid pGA22, in which the expression of the fusion gene was driven by the P_{lac} promoter, was induced with 1 mM IPTG during steady state growth. gal-gag110 protein accumulated in cells at concentrations that were estimated by Coomassie blue-stained SDS-PAGE to be as high as 40–50% of total protein content (not shown). After cell lysis and centrifugation most of the fusion protein was found in the precipitate, from which it was solubilized and quantitatively recovered with a denaturation–renaturation step. This procedure yielded a large amount of gal-gag110 (80–90% pure) which retained full β-galactosidase activity and was routinely used for HIV-1 protease assays of microbial fermentation broths. Pure gal-gag110 was obtained by affinity chromatography on TPEG-Sepharose, exploiting the binding properties of the β-galactosidase portion [27].

Fig. 2A shows the SDS-PAGE analysis of gal-gag110 fusion protein before and after incubation with rec m-binant HIV-1 protease. As incubation time increases the intact protein of about 130 kDa (lane b) is gradually replaced by the cleaved product (about 120 kDa, lanes d and e), as expected from the loss of an 80-amino acid fragment (Fig. 1). The cleavage reaction is strongly inhibited by pepstatin A (Fig. 2A, lane f), a known inhibitor of HIV-1 protease [28,29].

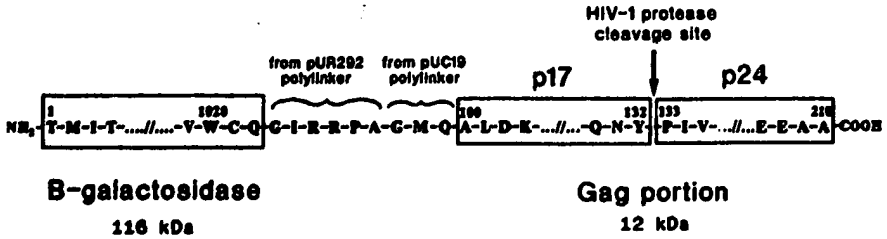

Fig. 1. Description f gal-gag110 fusion protein.

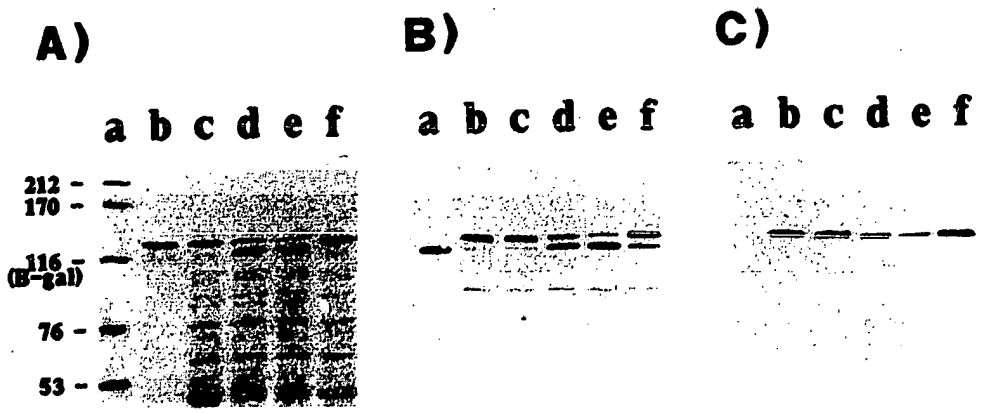

Fig. 2. Cleavage of gal-gag110 fusion protein by HIV-1 protease. (A) SDS-PAGE analysis. Renatured gal-gag110 (200 μg) was treated with 1 μl f crude HIV-1 protease preparation [24] in 100 μl of 0.2 M MES/NaOH buffer, pH 6.0, 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 1 mM PMSF at 37°C. Lanes: (a) molecular mass standards, indicated in kDa; (b) gal-gag110, non-treated; (c) gal-gag110 treated for 60 min with a crude extract from *E. coli* cells lacking the HIV-1 protease expression vector (control); (d) and (e) gal-gag110 treated with HIV-1 protease for 30 and 60 min respectively; (f) same as (e), but in the presence of 0.5 mM pepstatin A (Calbiochem). SDS-PAGE was performed in a Phast System apparatus (Pharmacia), using 7.5% acrylamide gels, β-ME as reducing agent and Coomassie blue for staining. (B) Western Blot analysis of the same gel using an anti-β-galactosidase monoclonal antibody (Boehringer Mannheim, Germany) followed by anti-mouse AuroProbe BL (Janssen, Belgium). Lane order as in (A). (C) Western Blot analysis of the same gel using an anti-p24 polyclonal antibody (Biochrom, Germany) and anti-sheep AuroProbe BL. Lane order as in (A). Both immunoblots were treated with Intense BL silver enhancement kit (Janssen).

A Western Blot analysis with two different antibodies c nfirms the identity of the protein bands. As expected, an anti-β-galactosidase antibody recognizes both uncleaved and cleaved gal-gag110 (Fig. 2B), while an anti-p24 polyclonal antibody binds only to the uncleaved protein (Fig. 2C), since in the cleaved fusion the p24 portion has been cut off by HIV-1 protease.

3.2. *Characterization of MAb 1G12*

The gal-gag110 fusion protein was used as immunogen to produce monoclonal antibodies. MAb 1G12, was selected for its ability to recognize the intact antigen but not β-galactosidase, as indicated by both ELISA and Western Blot experiments (not shown).

When MAb 1G12 was analyzed with a Western Blot f a total HIV-1 lysate, containing all viral proteins, only the Gag precursor, p55, was recognized (Fig. 3). Neither p24 or p17 were bound by the antibody. This indicates that the epitope recognized by MAb 1G12 is indeed localized in the Gag portion of gal-gag110, but is destroyed upon cleavage by HIV-1 protease. This str ngly suggests that the HIV-1 protease cleavage site is comprised in such an epitope region.

Experiments with MAb 1G12 immobilized on microtiter wells confirmed that the antibody binds only uncleaved and not HIV-1 protease-cleaved gal-gag110. In addition, its binding affinity was found to be suitable for an ELISA-type immunoassay, as indicated by the bservation that the amount of bound antigen was not significantly reduced after repeated washing cycles.

3.3. *Screening of microbial metabolites for HIV-1 protease inhibitors*

Using recombinant HIV-1 pr tease, gal-gag110 fusion protein and MAb 1G12, a solid-phase immunoassay was devised to test microbial metabolites for HIV-1 protease inhibitors (see section 2 for description of the assay).

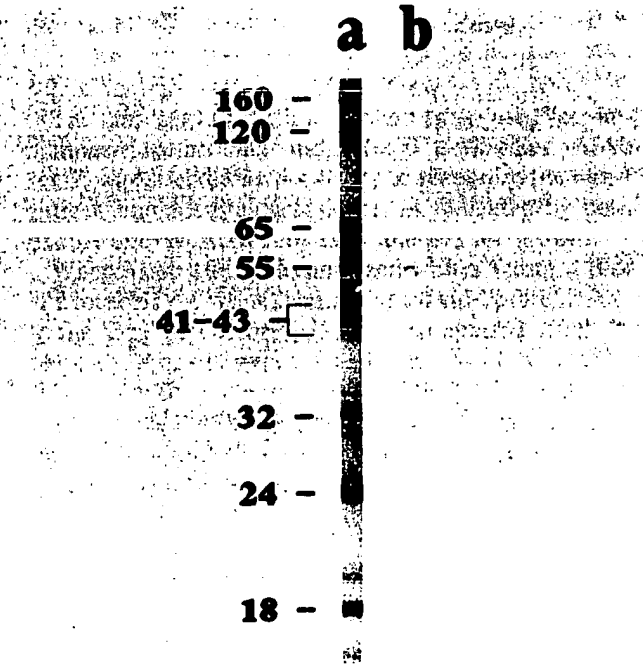

Fig. 3. Characterization of MAb 1G12 by Western Bl t analysis f total HIV-1 proteins. N vapath Immunoblot strips (Bio-Rad, Richm nd) were used. Lanes: (a) human serum (1:100 dil.) fr m an AIDS patient f ll wed by anti-human AuroProbe BL (Janssen); (b) MAb 1G12 (1 μg/ml) and anti-mouse AuroPr be BL (Janssen). In both cases silver enhancement (IntenSE BL, Janssen) was perf rmed.

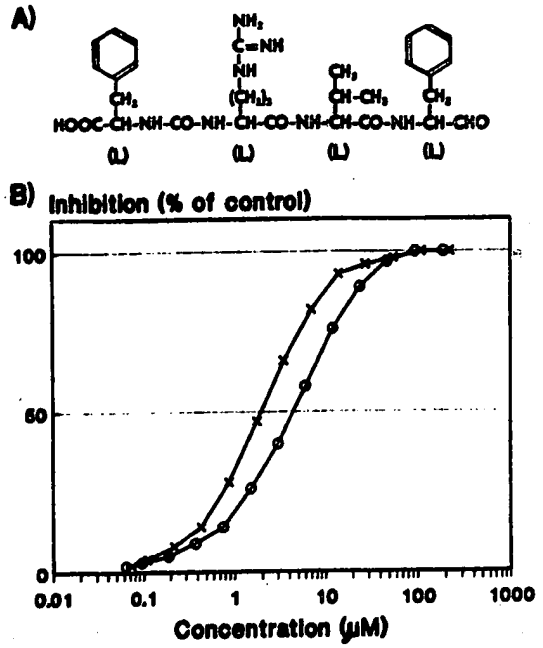

Fig. 4. (A) Chemical structure of α-MAPI [31]. (B) Dose-response plot showing the inhibition of cleavage of gal-gag110 by HIV-1 protease observed in the presence of the indicated concentrations of (x) α-MAPI or (○) pepstatin A (Calbiochem). The assay was performed essentially as described in section 2, in the presence of 5% DMSO. Determinations were from two separate experiments.

With this detection system about 12 000 fermentation broths were tested for the presence of HIV-1 protease inhibitors. These broths were derived mostly from Actinomycetes genera or fungal isolates. About 0.25% of them were found to contain significant and reproducible inhibitory activities against the viral enzyme. Among these activities one which showed good stability even after 1 h of incubation with horse or bovine serum was selected and the protease inhibitor was purified and characterized. The chemical structure determination allowed its identification as a peptide derivative of known structure, previously named α-microbial alkaline protease inhibitor, or α-MAPI (Fig. 4A) [30,31]. Fig. 4B shows the inhibition of the HIV-1 protease reaction observed at different concentrations of α-MAPI and pepstatin A. These two natural products display similar dose-response curves with 50% inhibition of substrate cleavage observed at 2.0 μM α-MAPI and 4.5 μM pepstatin A.

4. DISCUSSION

A novel solid-phase immunoassay has been developed to detect HIV-1 protease inhibitors in large numbers of biological samples. For such purpose the system described is fast and efficient and it has been used to screen about 12 000 fermentation broths. The most interesting aspect of this method is the ability to detect the presence of an HIV-1 protease inhibitor by the appearance of an enzymatic activity (i.e. β-galactosidase) instead of the disappearance of the protease activity. This provides an internal control: a positive response (colour) is obtained only when the protease reaction is inhibited. All other possible interfering activities (e.g. inhibition of MAb 1G12 binding, non-specific proteolysis of gal-gag110 or MAb 1G12, inhibition of β-galactosidase activity, etc.) would result in a negative response. Consequently, such an assay is particularly suitable for the screening of complex mixtures like microbial fermentation broths, where false positives are often a serious problem.

However, the detection system described here can be used to screen compounds from any origin for inhibitory activity on HIV-1 protease. The assay is also relatively unaffected by low concentrations (5–10%) of organic solvents like methanol, acetonitrile or DMSO, a feature particularly useful when it is used to monitor the purification of an inhibitory activity.

Using this detection system about 12 000 fermentation broths were screened and several positives were found. One of these inhibitory activities was purified and identified as α-MAPI [31]. This finding was largely unexpected since this compound, as expressed in its name, had been previously characterized as an inhibitor of alkaline proteases, with no activity on pepsin or other aspartic proteases [30]. Nevertheless, as shown in Fig. 4B, the inhibitory activity of α-MAPI on HIV-1 protease is comparable to pepstatin A, a characteristic inhibitor of aspartic proteases.

As shown in Fig. 4A, α-MAPI has a peptide-like structure with a C-terminal aldehyde group, like another natural product, tyrostatin, which has recently been reported to inhibit some acidic proteases [32]. In preliminary kinetic studies using purified HIV-1 protease and an HPLc assay based on a synthetic heptapeptide as substrate [3], α-MAPI was found to act as a non-competitive inhibitor (C. Tarnus, personal communication). These observations suggested the direct involvement of the C-terminal aldehyde group in the inhibition mechanism. Such a prediction was confirmed by the loss of inhibitory activity on HIV-1 protease observed after treating α-MAPI with permanganate (S. Stella, unpublished results) in conditions known to cause the selective oxidation of the aldehyde to carboxylic acid [31].

Studies are currently in progress to elucidate the mechanism of inhibition of HIV-1 protease by α-MAPI. At the same time other HIV-1 protease inhibitors found in microbial metabolites are being characterized, with the goal to find novel structures with improved in vitro and in vivo anti-HIV-1 properties.

Acknowledgements: We wish to thank A. Guindani and E. Corti for excellent technical assistance. We are also grateful to B. Harris for providing the recombinant HIV-1 protease, and C. Tarnus, K. Islam and M. Jung for helpful discussions.

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Nuclear access and action of notch in vivo.

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The Drosophila Notch (N) gene encodes a conserved single-pass transmembrane receptor that transduces extracellular signals controlling cell fate. Here, we present evidence that the intracellular domain of Notch gains access to the nucleus in response to ligand, possibly through a mechanism involving proteolytic cleavage and release from the remainder of the protein. In addition, our results suggest that signal transduction by Notch depends on the ability of the intracellular domain, particularly the portion containing the CDC10 repeats, to reach the nucleus and to participate in the transcriptional activation of downstream target genes.

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Nuclear Access and Action of Notch In Vivo

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Summary

The *Drosophila Notch* (*N*) gene encodes a conserved single-pass transmembrane receptor that transduces extracellular signals controlling cell fate. Here, we present evidence that the intracellular domain of Notch gains access to the nucleus in response to ligand, possibly through a mechanism involving proteolytic cleavage and release from the remainder of the protein. In addition, our results suggest that signal transduction by Notch depends on the ability of the intracellular domain, particularly the portion containing the CDC10 repeats, to reach the nucleus and to participate in the transcriptional activation of downstream target genes.

Introduction

Notch belongs to a conserved family of transmembrane receptors that transduce intercellular signals controlling cell fate (reviewed in Weinmaster, 1997; Greenwald, 1998). All of the members of this family are single-pass transmembrane proteins that respond to ligands of the Delta-Serrate-Lag2 (DSL) family and contain several conserved sequence motifs. The large extracellular domain contains many tandem repeats of an epidermal growth factor (EGF) motif as well as three copies of a "Lin-12/N repeat" (LNR). In addition, they contain intracellular domains that include a block of six CDC10 (or ankyrin) repeats, one or two nuclear localization signals (NLS), a homopolymer repeat of glutamine (an "OPA" domain), and a proline-glutamate-serine-threonine-rich "PEST" domain. However, the intracellular domains of Notch proteins lack any recognizable catalytic motif, and it remains unclear how they transduce extracellular signals.

Truncated forms of Notch that consist of only the intracellular domain have constitutive transducing activity (Lieber et al., 1993; Struhl et al., 1993) and localize predominantly in the nucleus (Fortini et al., 1993; Lieber et al., 1993; Struhl et al., 1993; Kopan et al., 1994). Hence, it has been proposed that interactions between ligand and the extracellular domain of Notch induce the release of the intracellular domain from the membrane and that the intracellular domain translocates to the nucleus, where it transduces N signals by regulating the transcription of downstream target genes (Lieber et al., 1993; Struhl et al., 1993; Kopan et al., 1994).

The possibility of such a direct mechanism of signal transduction remains controversial. On the positive side, Notch cleavage products that include portions of the Notch intracellular domain have been identified in tissue culture cells and in whole animal extracts (e.g., Aster et al., 1994; Kopan et al., 1996; Blaumueller et al., 1997). Moreover, portions of the Notch intracellular domain have been shown to associate physically with the Suppressor of Hairless (Su[H]) protein, a sequence-specific DNA-binding protein, which appears responsible for activating the transcription of downstream target genes in response to Notch receptor activity (reviewed in Honjo, 1996; Weinmaster, 1997).

On the negative side, all attempts to obtain direct evidence for ligand-dependent nuclear access of the Notch intracellular domain have failed, despite the existence of antisera specific for epitopes within the intracellular domain and the ability to examine altered forms of Notch (e.g., lacking portions of the extracellular domain including the LNRs) that have constitutive transducing activity (Fehon et al., 1991; Lieber et al., 1993; Rebay et al., 1993). Also, there is no direct evidence that the productive interaction between Notch and Su(H) occurs within the nucleus. Indeed, analyses of the physical relationships between Notch and Su(H) protein in tissue culture and in vivo have led to proposals in which Notch tethers Su(H) at the cell surface, releasing and perhaps modifying Su(H) in response to ligand (Fortini and Artavanis-Tsakonas, 1994; Lecourtois and Schweisguth, 1995; Kopan et al., 1996; Roehl et al., 1996).

Here, we describe experiments designed to test whether the Notch intracellular domain normally gains access to the nucleus in response to ligand and whether it acts within the nucleus to regulate the transcription of downstream target genes. Our results provide evidence that such access occurs, possibly by a mechanism involving proteolytic cleavage that leads to the release of the intracellular domain from the rest of the protein. In addition, they support the hypothesis that the intracellular domain of Notch, and particularly the CDC10 repeats, transduce Notch signals by acting within the nucleus to activate gene transcription.

Results

Evidence for Nuclear Access of the Notch Intracellular Domain in Vivo

As noted in the Introduction, attempts to detect the physical presence of intracellular portions of Notch in the nucleus by conventional immunological or biochemical means have generally been unsuccessful, even in cells in which the receptor is active. We have therefore employed a potentially more sensitive approach in which the chimeric transcription factor Gal4-VP16 (GV) is inserted at various positions in otherwise wild-type Notch protein (Figure 1) and the resulting N^+-GV proteins expressed under heat shock control in embryos that also carry a *UAS-lacZ* transgene (Experimental Procedures). The Gal4-VP16 protein contains the DNA-binding domain of the yeast Gal4 transcription factor coupled to

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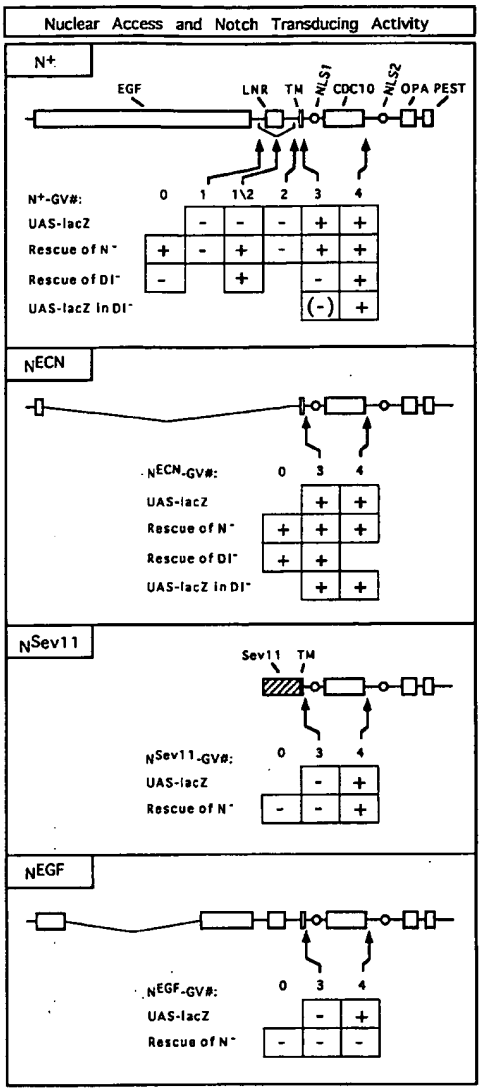

Figure 1. Summary of Experiments Assaying Nuclear Access and Transducing Activity of Notch

The general structural features of Notch are diagrammed in the top panel with the EGF repeats, Lin12/Notch repeats (LNR), transmembrane domain (TM), NLSs, CDC10 repeats, OPA repeats, and PEST sequences indicated. The Gal4-VP16 domain was inserted at four sites (arrows) and the resulting N^+-GV proteins designated by the insertion site (1–4; 0 = no Gal4-VP16 insertion; 1\2 = an insertion of Gal4-VP16 in place of the domain normally positioned between sites 1 and 2). The N^{ECN}, N^{Sev11}, and N^{EGF} derivatives of these proteins are diagrammed in the panels underneath (details of these constructs are given in Table 1; note that the N^{Sev11} derivative is composed of the extracellular and transmembrane domains of the Sev11 protein [hatched and black, respectively] joined to the intracellular domain of Notch). All of the derivatives shown were tested for nuclear access of the inserted Gal4-VP16 domain by assaying their ability to activate transcription of a *UAS-lacZ* target gene. In addition, all were tested for their ability to activate the Notch transduction pathway by assaying whether they could rescue epidermal development in N^{ts1} arm^{YD35} embryos. Finally, selected derivatives were tested for ligand-dependent nuclear access and Notch transducing activity by assaying their ability to activate *UAS-lacZ* expression in Dl^- versus *hsp70-Dl* Dl^- embryos and to rescue epidermal development in Df^{K43} hh^{10E} embryos. The assays used (see Experimental Procedures) generally yielded qualitatively distinct results as illustrated in Figures 2, 5, and 6, except for *UAS-lacZ* expression activated by N^+-GV3 protein in Dl^- embryos. In this case, rare *UAS-lacZ*–expressing cells were observed (Figure 2) and the result indicated as "(–)" rather than "–".

the transcriptional activating domain of the viral VP16 protein (Sadowski et al., 1988). The *UAS-lacZ* gene contains four copies of the UAS-binding site for Gal4 and is transcribed in response to Gal4 as well as the Gal4-VP16 protein in *Drosophila melanogaster* (Fischer et al., 1988; data not shown). We reasoned that expression of the *UAS-lacZ* gene would provide a sensitive assay for nuclear access of the inserted Gal4-VP16 domain and hence for events that lead to nuclear import of the Notch intracellular domain.

The Gal4-VP16 coding sequence was inserted at either of two positions in the intracellular domain: just carboxy-terminal to the transmembrane domain to generate the chimeric protein N^+-GV3 and after the domain containing the CDC10 repeats to generate the chimeric protein N^+-GV4 (Figure 1). Heat shock–induced expression of each of these proteins during embryogenesis caused expression of the *UAS-lacZ* gene in the ventral ectoderm and the developing central nervous system, as well as in other tissues (Figures 2 and 6; data not shown). Cells in each tissue appeared to respond in a salt and pepper fashion similar to that caused by low level expression of the Gal4-VP16 protein alone (data not shown). Our initial assays were performed using a severe heat shock (37°C for 1 hr, followed by a 2 hr recovery), which generates levels of N^+-GV protein that are similar to that of endogenous Notch (data not shown). However, mild heat shock (e.g., 33°C for 60 min, followed by a 2 hr recovery) resulted in detectable expression of the *UAS-lacZ* gene, even though the level of N^+-GV protein is severalfold lower than the level of endogenous Notch (data not shown). We have examined the subcellular localization of the Gal4 DNA-binding domain inserted in both proteins, as well as in all of the remaining Gal4 derivatives shown in Figures 1 and 4, in the embryonic ectoderm. In all cases, the Gal4 DNA-binding domain appears to be localized predominantly at the cell periphery (data not shown), as is the case for both extracellular and intracellular epitopes of the endogenous Notch protein (Fehon et al., 1991).

We also assayed the ability of these chimeric proteins to provide Notch transducing activity. Specifically, we have asked whether their expression can suffice to rescue formation of the ventral epidermis in N^- embryos in which all cells of the ventral ectoderm would otherwise develop as neuroblasts. To facilitate the analysis, we used arm^- N^- embryos: when Notch function is restored in such embryos, they secrete a ventral cuticle that displays the Armadillo segmentation phenotype allowing them to be identified unambiguously (Lieber et al., 1993; Figure 5). For both the N^+-GV3 and N^+-GV4 proteins, we observed rescue (data not shown).

To test whether nuclear access of the inserted Gal4-VP16 domain depends on its being located within the intracellular domain, we assayed the activities of the

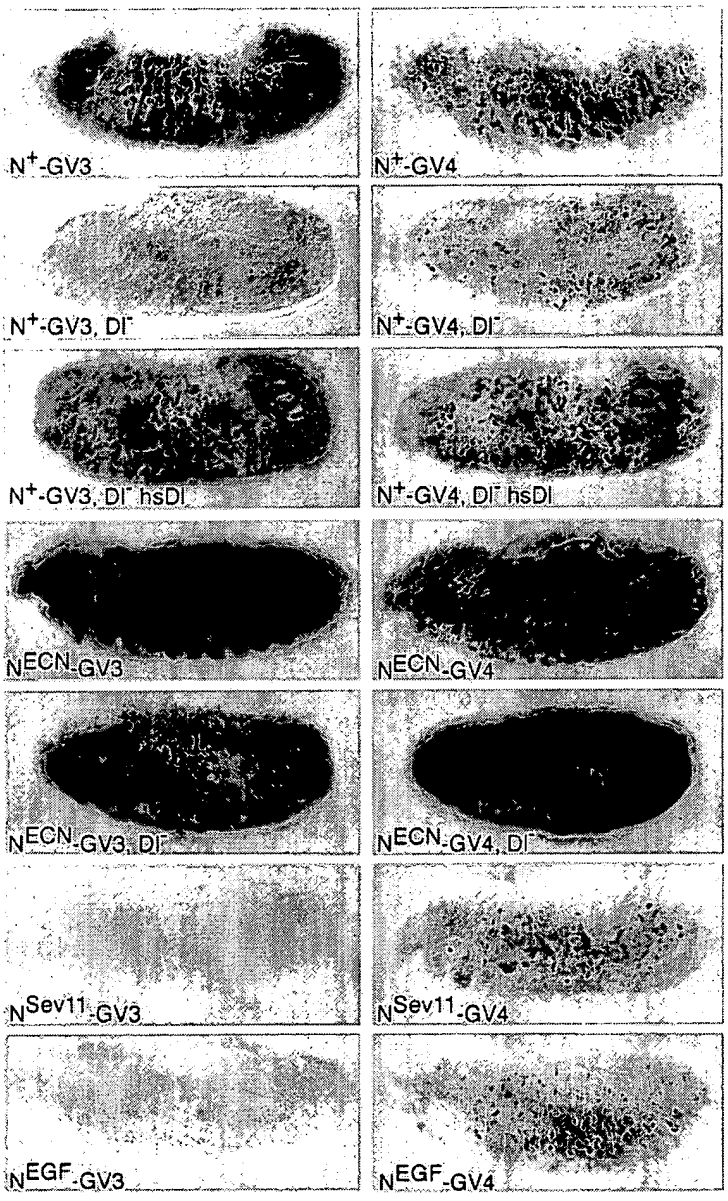

Figure 2. Ligand Dependence of Notch Nuclear Access

Embryos carrying the *UAS-lacZ* reporter gene and expressing the wild-type, ECN, Sev11, and EGF derivatives of N-GV3 and N-GV4 protein (Figure 1) are shown stained for β-gal protein expression. For the N-GV3 derivatives, only the wild-type and ECN forms induce β-gal expression. Moreover, the wild-type form does so in a ligand-dependent fashion, as indicated by the relative absence of β-gal expression in *Dl*⁻ embryos compared to *Dl*⁺ embryos and to *Dl*⁻ embryos supplemented with ectopic Delta expressed under heat shock control (the few cells that express β-gal in *Dl*⁻ embryos may reflect a response of the N⁺-GV3 protein to another ligand, possibly Ser). In contrast, the ECN form induces similar amounts of β-gal expression in *Dl*⁻ embryos compared to *Dl*⁺ embryos, indicating that it does so in a ligand-independent fashion. For the N-GV4 derivatives, all induce β-gal expression even in the absence of ligand (N⁺-GV4 in *Dl*⁻ embryos) or when portions of the extracellular domain normally required for ligand-dependent activation of Notch are deleted (N^{Sev11}-GV4 and N^{EGF}-GV4). However, the ability of N⁺-GV4 protein to induce β-gal expression retains some dependence on ligand (compare β-gal expression in *Dl*⁻ with *Dl*⁻ *hsDl* embryos). Here, as in Figure 6, all embryos are staged around the end of germband shortening, with anterior to the left; similar results were obtained in embryos staged as early as the completion of germband extension and as late as the completion of dorsal closure.

chimeric proteins N⁺-GV1 and N⁺-GV2, which contain Gal4-VP16 insertions in either of two sites in the extracellular domain, just before the LNR domain and just before the transmembrane domain (Figure 1). In contrast to the results obtained with the N⁺-GV3 and N⁺-GV4 proteins, no *UAS-lacZ* expression was observed in embryos expressing N⁺-GV1 or N⁺-GV2 protein (data not shown). However, neither the N⁺-GV1 nor the N⁺-GV2 protein was able to restore epidermal development in *arm*⁻ *N*⁻ embryos (Figure 1), indicating that the chimeric protein cannot function normally to transduce Notch signals. We therefore expressed another chimeric protein, N⁺-GV1\2, in which the Gal4-VP16 coding sequence was inserted in place of the LNR domain. As previously shown, deletion of the LNR domain renders Notch constitutively active (Lieber et al., 1993), and we find that expression of the N-GV1\2 chimeric protein can rescue the formation of ventral epidermis not only in *N*⁻ embryos but also in *Dl*⁻ embryos (Figure 1). Nevertheless, the N⁺-GV1\2 chimeric protein does not drive *UAS-lacZ* expression (Figure 1). We conclude that the Gal4-VP16 domain present in the N⁺-GV1\2 protein does not have access to the nucleus, even though the chimeric receptor is constitutively active.

Thus, Gal4-VP16 insertions within the Notch intracellular domain, both amino-terminal and carboxy-terminal to the CDC10 domain, appear to have access to the nucleus in vivo, in contrast to Gal4-VP16 insertions in the extracellular domain, which do not. In principle, this access could be afforded by translocation of the entire receptor from the membrane to the nucleus. Alternatively, access may be afforded by one or more cleavage

events that occur amino-terminal to the inserted Gal4-VP16 domain and allow release and nuclear import of carboxy-terminal portions of the protein.

Evidence for Ligand-Dependent Nuclear Access of Notch

We have performed two sets of experiments to determine whether nuclear access of the Gal4-VP16 domain in N^+-GV3 protein depends on ligand.

In the first set of experiments, we have assayed *UAS-lacZ* expression in embryos expressing a series of N-GV3 proteins that have alterations of the extracellular domain, which block their ability to respond to ligand or which render them constitutively active irrespective of ligand (Figure 1). One derivative, referred to as N^{ECN}-GV3, lacks virtually all of the extracellular domain, including all 36 EGF repeats and the LNR domain. N^{ECN} protein has previously been shown to have ectopic transducing activity, suggesting that it is constitutively active irrespective of ligand (Fortini et al., 1993; Rebay et al., 1993). A second, referred to as N^{EGF}-GV3, consists of a deletion of EGF repeats 4-26: like other forms of Notch that have internal deletions of the EGF region (Lieber et al., 1993; Rebay et al., 1993), N^{EGF} protein cannot rescue the absence of endogenous Notch activity (Figure 1), indicating that it is unable to respond to ligand. The third, referred to as N^{Sev11}-GV3, lacks the entire extracellular domain as well as the transmembrane domain of Notch and has in its place the extracellular and transmembrane domains of Sev11, a truncated form of the receptor tyrosine kinase Sevenless (Basler et al., 1991). Like N^{EGF} protein, N^{Sev11} protein also appears unable to transduce ligand (Figure 1).

The main result we have obtained is that the N^+-GV3 and N^{ECN}-GV3 proteins can activate *UAS-lacZ* transcription and, in addition, can provide Notch transducing activity to otherwise *N*$^-$ embryos. In contrast, the N^{EGF}-GV3 and N^{Sev11}-GV3 proteins can do neither (Figures 1 and 2). Thus, nuclear access of the Gal4-VP16 domain inserted in N-GV3 proteins correlates with Notch transducing activity: forms of N-GV3 protein that behave as if they cannot transduce Notch signals do not allow access, whereas forms that have ligand-dependent or constitutive transducing activity do allow access.

In the second set of experiments, we have assayed whether nuclear access and Notch transducing activity associated with the N^+-GV3 and N^{ECN}-GV3 proteins depend on Delta (Dl), the primary ligand for activating Notch in embryos (reviewed in Weinmaster, 1997; Greenwald, 1998). To test whether nuclear access depends on Delta, we compared the ability of the N^+-GV3 and N^{ECN}-GV3 proteins to activate *UAS-lacZ* expression in heat-shocked *Dl*$^-$ embryos versus heat-shocked *Dl*$^-$ embryos in which Delta expression is restored by a *hsp70-Dl* transgene (see Experimental Procedures). For the N^+-GV3 protein, we find that only very few cells show *UAS-lacZ* expression in the absence of Delta (Figure 2). However, in the presence of heat shock–induced Delta, *UAS-lacZ* is widely expressed (Figure 2), indicating that the response is ligand-dependent. By contrast, the N^{ECN}-GV3 protein activates *UAS-lacZ* expression irrespective of Delta (Figure 2).

To test whether the Notch transducing activities of the N^+-GV3 and N^{ECN}-GV3 proteins are Delta-dependent, we asked whether heat shock–induced expression of either protein can rescue epidermal differentiation in *Dl*$^-$ embryos (for these experiments, the *Dl*$^-$ mutation was linked with a *hh*$^-$ mutation so that rescued embryos could be unambiguously identified by the Hedgehog segmentation phenotype; see Experimental Procedures). We find that expression of the N^+-GV3 protein fails to rescue epidermal differentiation in *Dl*$^-$ *hh*$^-$ embryos, in contrast to expression of the N^{ECN}-GV3 protein, which does (Figure 1; data not shown). Consequently the transducing activity of N^+-GV3 protein, but not N^{ECN}-GV3 protein, appears to be ligand-dependent.

Thus, both sets of experiments provide evidence that nuclear access of the Notch intracellular domain, as assayed by ability of the Gal4-VP16 domain of N-GV3 proteins to activate *UAS-lacZ* expression, depends on ligand and correlates with Notch transducing activity.

Evidence for Nuclear Access of Notch in the Absence of Ligand

We also analyzed nuclear access of the Gal4-VP16 domain in N-GV4 proteins, using the same tests for ligand dependence employed for the N-GV3 proteins. As shown in Figure 2, the main result we have obtained is that nuclear access of this domain, which is inserted carboxy-terminal to the CDC10 repeats, appears to depend on both ligand-dependent and ligand-independent mechanisms. For example, the N^{Sev11}-GV4 and N^{EGF}-GV4 proteins can activate *UAS-lacZ* expression, even though they should not be able to respond to ligand. Similarly, the N^+-GV4 protein can activate the *UAS-lacZ* gene in many cells in *Dl*$^-$ embryos, despite the absence of Delta protein. Both sets of results indicate that carboxy-terminal portions of the Notch intracellular domain that include the GV4 insertion have access to the nucleus even in the absence of normal ligand stimuation. However, we also find evidence that ligand stimulates nuclear access of the Gal4-VP16 domain in N^+-GV4 protein. In particular, we consistently observe that more cells express the *UAS-lacZ* gene in heat-shocked *hsp70-N*$^+$*-GV4 Dl*$^-$ embryos when these embryos carry the *hsp70-Dl* transgene compared to when they do not.

Thus, at least some portions of the Notch intracellular domain appear to gain access to the nucleus in a ligand-independent fashion, possibly as a consequence of proteolytic cleavages that occur carboxy-terminal to the site of the GV3 insertion. We note that this ligand-independent access is not likely to be productive in terms of normal Notch signaling because wild-type Notch has no transducing activity in the absence of ligand, and both the N^{EGF} and N^{Sev11} proteins similarly lack transducing activity even in the presence of ligand (Figure 1; see Discussion).

Transducing Activity of the CDC10 Domain of Notch Depends on Access to the Nucleus

The ability of Notch to transduce extracellular signals depends critically on the integrity of the CDC10 repeats within the intracellular domain (Greenwald and Seydoux, 1990; Kodoyianni et al., 1992; Lieber et al., 1993; Rebay

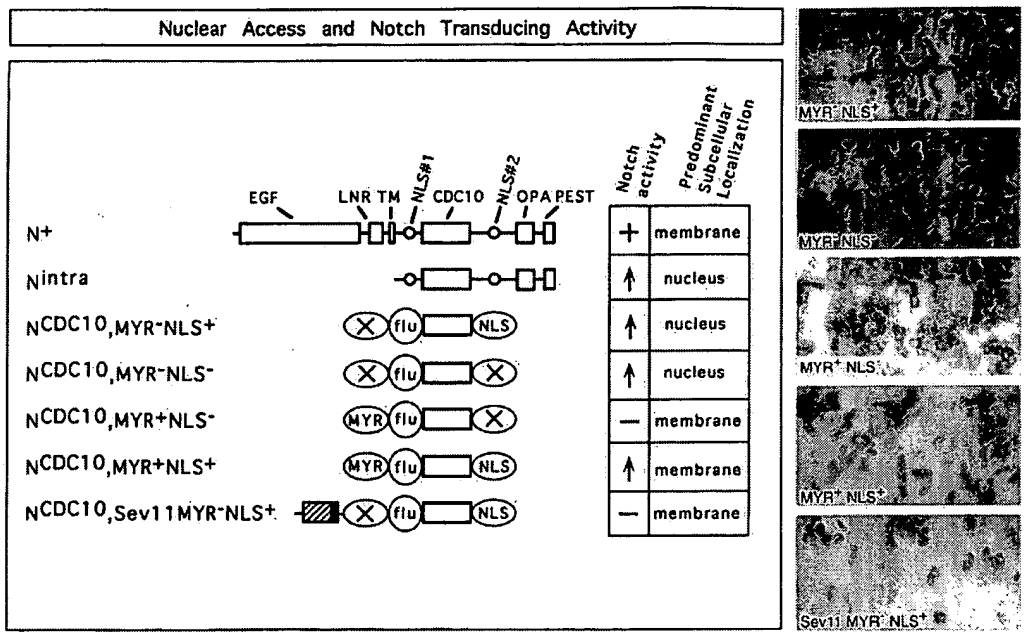

Figure 3. Predominant Subcellular Localization and Notch Transducing Activity of N^{CDC10} Proteins

The structures of Notch, N^{intra}, and the various derivatives of Flu-tagged N^{CDC10} proteins are diagrammed as in Figure 1 (the results for Notch and N^{intra} have been published previously; Struhl et al., 1993). The top four N^{CDC10} derivatives are identical except for a single G→A substitution, which inactivates the MYR signal in the MYR^- derivatives, and adjacent K→T substitutions, which inactivate the NLS signal in the NLS^- derivatives. The last derivative, N^{CDC10},Sev11MYR^-NLS^+ (bottom) is composed of the extacellular and transmembrane domains of the Sev11 protein fused immediately upstream of the N^{CDC10},MYR^-NLS^+ protein. All of the proteins shown were tested for Notch transducing activity by examining whether they could rescue epidermal development in *arm*$^-$ *N*$^-$ embryos (assay 1), whether their expression could rescue epidermal development in *Dl*$^-$ *hh*$^-$ embryos (assay 2), and whether their expression could suppress neuroblast segregations in early *N*$^+$ embryos (assay 3). Wild-type Notch protein rescues the absence of the endogenous gene (1), but does not rescue the absence of Delta (2) or cause an anti-neurogenic phenotype (3): it is scored as "+". In contrast, the remaining proteins either have all three activities (indicating that they have intrinsic activity, scored as ("!"), or none of these activities (scored as "−"). In addition, the predominant subcellular localization of each of the N^{CDC10} derivatives was assayed using antisera against the CDC10 (in *N*$^-$ embryos) and Flu epitopes. The ventral ectoderms of embryos having clones of cells expressing each of the five forms of N^{CDC10} proteins are shown on the right stained for expression of the Flu epitope (embryos are staged at the end of the early phase of germband extension). Note that the two derivatives that are found predominantly in the nucleus have intrinsic Notch transducing activity, in contrast to two of the three derivatives that appear to be largely excluded from the nucleus and lack detectable activity. The remaining derivative, N^{CDC10},MYR^+NLS^+, is exceptional in that it retains intrinsic transducing activity even though it appears to be predominantly membrane associated. However, this protein differs from the N^{CDC10},MYR^+NLS^- protein in that it carries a wild-type rather than a mutant NLS sequence. We infer that the presence of the active NLS signal in this protein allows a small amount to reach the nucleus despite the presence of the MYR^+ signal, accounting for its intrinsic transducing activity.

et al., 1993), and in at least one case, a relatively small fragment of the intracellular domain that contains these repeats has intrinsic transducing activity (Roehl and Kimble, 1993). We have therefore asked whether the intrinsic transducing activity of such a fragment of Notch, termed N^{CDC10}, depends on whether it can gain access to the nucleus.

As diagrammed in Figure 3, we have coupled a polypeptide containing the N^{CDC10} fragment and two copies of the Flu-epitope tag (Wilson et al., 1984) with wild-type or mutated versions of the myristylation signal (MYR) of *Drosophila* Src (Cross et al., 1984; Simon et al., 1985) at the amino terminus and of a nuclear localization signal (NLS) from SV40 T antigen (Kalderon et al., 1984) at the carboxy terminus to create four tagged forms of the CDC10 domain. In principle, these should be targeted to membranes (MYR^+NLS^-) or the nucleus (MYR^-NLS^+), or to neither (MYR^-NLS^-) or both (MYR^+NLS^+). These four proteins are identical except for changes in a single amino acid (Gly or Ala) within the MYR signal and in two adjacent amino acids (Lys-Lys or Thr-Thr) within the NLS.

Each of the four proteins was expressed using a combination of the Gal4/UAS (Fischer et al., 1988; Brand and Perrimon, 1993) and Flp-out (Basler and Struhl, 1994) techniques to create embryos in which all, or only some, cells express the tagged protein (see Experimental Methods) and the results of these experiments shown in Figure 3. The MYR^-NLS^+ protein is localized predominantly in the nucleus and has intrinsic Notch transducing activity, as indicated by its ability to block neuroblast segregations in wild-type embryos and to rescue epidermal differentiation in *arm*$^-$ *N*$^-$ and in *Dl*$^-$ *hh*$^-$ embryos. Similarly, MYR^-NLS^- protein accumulates predominantly in the nucleus, despite the absence of an active NLS, and has intrinsic signal-transducing activity. In contrast, the MYR^+NLS^- protein appears to be excluded from the nucleus and lacks detectable Notch transducing activity as it fails to rescue *arm*$^-$ *N*$^-$ embryos or to block neuroblast segregations. In the case of the

MYR$^+$NLS$^+$ protein, most of the protein accumulates outside of the nucleus, indicating that the MYR$^+$ signal predominates over the NLS$^+$ signal. Nevertheless, this protein retains Notch transducing activity, perhaps because the presence of an active NLS allows a small amount to gain access to the nucleus despite the presence of an active MYR signal. To test this possibility, we created a fifth chimeric protein in which the Sev11 extracellular and transmembrane domains were fused immediately upstream of the MYR$^-$NLS$^+$ protein. The resulting protein, Sev11MYR$^-$NLS$^+$, has a conventional signal sequence at its amino terminus and should be inserted into the membrane as it is synthesized. As a consequence, it should remain stably membrane associated, despite the presence of an active NLS at its carboxy terminus. As expected, the Sev11MYR$^-$NLS$^+$ protein appears to be excluded from the nucleus. Moreover, it lacks detectable Notch transducing activity, in contrast to MYR$^+$NLS$^+$ protein.

Thus, a discrete CDC10-containing portion of the Notch intracellular domain has intrinsic transducing activity. However, this transducing activity appears to require that the protein has access to the nucleus, as it is enhanced by nuclear targeting and abolished by membrane targeting. Because the CDC10 repeats are essential for signal transduction in the context of the wild-type protein, we interpret these results as evidence that transduction by Notch normally requires access of the CDC10 repeats to the nucleus.

Evidence for Transcriptional Regulation by the Notch Intracellular Domain

One reason why Notch signal transduction may depend on nuclear access of the CDC10 domain is that this domain is directly involved in regulating transcription in response to ligand. To test this possibility, we have assayed the consequences of inserting protein domains that have well-characterized roles in mediating transcriptional activation or repression into the intracellular domain of otherwise intact Notch. We reasoned that if the Notch intracellular domain normally participates in transcriptional regulation, adding such activating or repressing domains might have opposite effects on the regulation of downstream target genes, yielding phenotypes corresponding to gain or loss of activity of the Notch pathway.

To assay the effects of inserting an activator domain, we compared the consequences of expressing N$^+$-GV4 protein, which contains the transcriptional activating domain of VP16, with those of expressing a control chimeric protein, N$^+$-G4, which contains the Gal4 DNA-binding domain at the same site but lacks the VP16 activation domain (Figure 4). We find that both proteins have Notch transducing activity, as indicated by their ability to rescue the neurogenic phenotype of *arm*$^-$ *N*$^-$ embryos (Figures 4 and 5; data not shown). Moreover, both also are capable of directing *UAS-lacZ* expression in similar numbers of cells, although the level of expression is significantly lower in the case of N$^+$-G4 (Figures 4 and 6). However, two lines of evidence indicate that the added presence of the VP16 domain in N$^+$-GV4 protein renders it constitutively active in terms of Notch signal transduction, in contrast to N$^+$-G4 protein, which lacks this domain and is ligand-dependent.

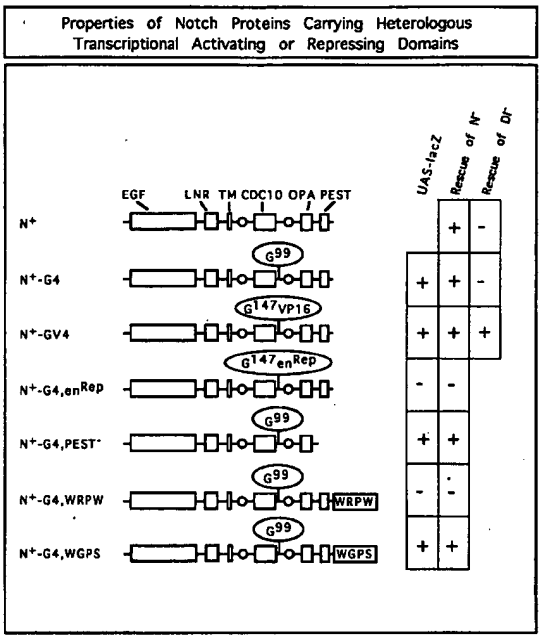

Figure 4. Summary of Experiments Providing Evidence for a Direct Involvement of the Notch Intracellular Domain in Transcriptional Regulation

A series of N$^+$-G4 derivatives that carry activator (VP16) or repressor (en^{Rep},WRPW) motifs, as well as appropriate control derivatives, are diagrammed on the left, and the results of assays for nuclear access and Notch transducing activity (see Figure 1; Experimental Procedures) are indicated on the right (see also Figures 5 and 6). G99 refers to the presence of the first 99 amino acids of Gal4 protein, which contain principally the DNA-binding domain. G147 refers to the presence of the first 147 amino acids of Gal4 protein; these additional amino acids are present in the Gal4-VP16 domain inserted in the N-GV proteins diagrammed in Figure 1.

First, we tested whether heat shock–induced expression of either N$^+$-G4 or N$^+$-GV4 protein can suffice to rescue the formation of ventral epidermis in *Dl*$^-$ *hh*$^-$ embryos. We observe that expression of the N$^+$-GV4 protein, but not N$^+$-G4, has rescuing activity (Figures 4 and 5; data not shown). Second, we tested whether the expression of either protein can block neuroblast segregations in embryos, an assay for constitutive Notch transducing activity (Struhl et al., 1993). We find that heat shock–induced expression of N$^+$-GV4 protein represses neuroblast segregations, both in wild-type and *Dl*$^-$ embryos (data not shown). In contrast, we could not detect an effect of heat shock–induced expression of N$^+$-G4 protein on these segregations.

Further evidence that insertion of the VP16 activator domain can cause constitutive activity of Notch comes from comparing the Notch transducing activities of the N^{Sev11}, N^{Sev11}-GV3, and N^{Sev11}-GV4 proteins (Figure 1). None of these proteins should be able to respond to ligand, owing to the substitution of the extracellular and transmembrane domains of the Sev11 protein for those of Notch. Nevertheless, the presence of the Gal4-VP16 domain inserted in N^{Sev11}-GV4 protein allows this protein to rescue epidermal development in *arm*$^-$ *N*$^-$ embryos, correlating with the ability of the Gal4-VP16 domain to

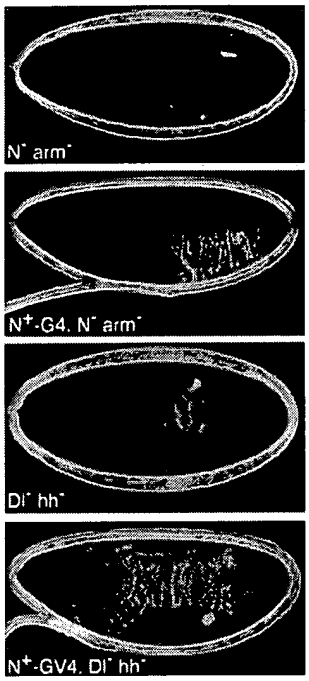

Figure 5. Rescue of Epidermal Differentiation in *arm⁻ N⁻* and *Dl⁻ hh⁻* Embryos by N⁺-G4 and N⁺-GV4 Proteins

In *arm⁻ N⁻* and *Dl⁻ hh⁻* embryos, virtually all ectodermal cells differentiate as neural tissue at the expense of epidermis, leading to absence of cuticular structures. However, epidermal differentiation is restored in *arm⁻ N⁻* embryos that express N⁺-G4 protein and in *Dl⁻ hh⁻* embryos that express N⁺-GV4 protein. The *arm⁻* and *hh⁻* mutations are linked, respectively, to the *N⁻* and *Dl⁻* mutations and cause distinctive "lawn" phenotypes of disorganized ventral hairs, confirming the genotypes of the "rescued" embryos.

gain access to the nucleus even in the absence of normal ligand interactions (Figure 1). One interpretation of this finding is that all three proteins are cleaved carboxy-terminal to the Sev11 transmembrane domain, but only intracellular cleavage products derived from the N^{Sev11}-GV4 protein include the VP16 activation and hence acquire Notch transducing activity. Extending this intepretation to the constitutive activity of N⁺-GV4 protein, we suggest that addition of the VP16 activator domain confers transducing activity to intracellular cleavage products of Notch that arise in the absence of ligand stimulation and would otherwise lack activity. We note that the added presence of the VP16 activation domain is not sufficient to restore Notch transducing activity in the case of N^{EGF}-GV4 protein. Nevertheless, this protein can activate *UAS-lacZ* expression, indicating that the Gal4-VP16 domain does have access to the nucleus (Figures 1 and 2). It is possible that N^{EGF} proteins may be processed differently than N^{Sev11} or N⁺ proteins, leading to intracellular cleavage products that lack Notch transducing activity even when coupled to a VP16 activating domain.

To assay the effects of inserting a repressor domain, we used two different motifs: an alanine-rich portion of the homeodomain protein Engrailed (en^{Rep}) and the WRPW tetrapeptide that is present at the C terminus of basic helix-loop-helix repressor proteins such as Hairy

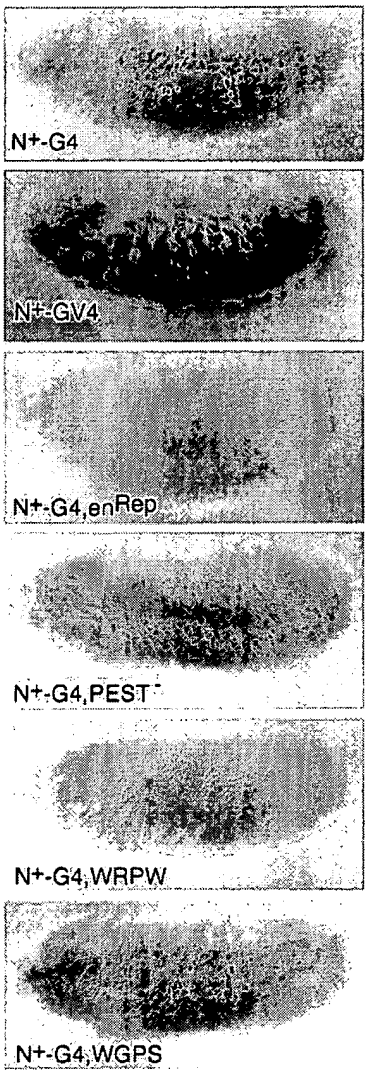

Figure 6. Regulation of *UAS-lacZ* Expression by N⁺-G4 Proteins Carrying Heterologous Transcriptional Activating or Repressing Domains

Embryos carrying the *UAS-lacZ* reporter gene and expressing the various derivatives of N⁺-G4 protein diagrammed in Figure 4 are shown stained for β-gal protein expression. The presence of just the DNA-binding domain of Gal4 in N⁺-G4 protein leads to moderate levels of β-gal expression. The further addition of the VP16 activation domain enhances this level of expression, while the addition of the en^{Rep} or Hairy (WRPW) repression domains completely blocks this expression. By contrast, the WGPS and PEST derivatives of N⁺-G4 protein behave similarly to the canonical N⁺-G4 protein.

and mediates transcriptional repression at least in part by recruiting the protein Groucho (Gro) (reviewed in Fisher and Caudy, 1998). In the first case, the en^{Rep} domain was inserted in place of the VP16 domain in N⁺-GV4 protein to generate the protein N⁺-G4,en^{Rep}; in the second case, the WRPW peptide was added to the end of N⁺-G4 protein to create the protein N⁺-G4,WRPW (Figure 4). As controls for the N⁺-G4,WRPW experiment we also examined the consequences of adding a mutated form of this peptide, WGPS (to generate the protein

N^+-G4,WGPS), or deleting the C-terminal 80 amino acids of Notch (to generate the protein N^+-G4,PEST$^-$). We find that both the N^+-G4,en^{Rep} and N^+-G4,WRPW proteins lack detectable Notch signal–transducing activity, as indicated by their failure to rescue epidermal development in *arm*$^-$ *N*$^-$ embryos (Figure 4; data not shown). In addition, they are unable to activate expression of the *UAS-lacZ* gene (Figures 4 and 6). In contrast, the N^+-G4,WGPS and N^+-G4,PEST$^-$ proteins behave similarly to N^+-G4: both have Notch signal–transducing activity and can direct *UAS-lacZ* expression (Figures 4 and 6; data not shown).

Thus, adding well-defined transcriptional activating or repressing domains to intact Notch has opposing effects on Notch signal transduction, causing gain or loss of activity, respectively. In a corresponding fashion, they also cause the enhancement or loss of *UAS-lacZ* expression mediated by a Gal4 DNA-binding domain inserted within Notch. We interpret these results as evidence that Notch signal transduction normally involves the direct participation of the intracellular domain in activating the transcription of downstream target genes.

Discussion

Eukaryotic cells employ many strategies to link the reception of extracellular signals to changes in gene expression. In general, these strategies depend on transmembrane receptors, which receive signals at the cell surface and modify one or more intracellular effector proteins that transduce these signals to the nucleus. The same mechanism may also apply for Notch signal transduction. In this case, interactions between ligand and Notch at the cell surface would modify an effector such as the DNA-binding protein Su(H), which then translocates to the nucleus and regulates gene expression. However, the Notch intracellular domain contains conserved nuclear localization signals and, when expressed on its own, accumulates predominantly in the nucleus and has constitutive transducing activity. These observations have suggested an alternative and unusual mechanism for Notch signal transduction. In this case, ligand binding to the extracellular domain of the receptor would induce translocation of the intracellular domain to the nucleus, where it acts, in association with Su(H) or other DNA-binding proteins, to regulate transcription.

As outlined in the Introduction, all previous attempts to obtain direct evidence for ligand-dependent nuclear access of the Notch intracellular domain have failed. Hence, if Notch transduces extracellular signals through a mechanism that depends on nuclear import of the intracellular domain, one would have to argue that the amount of protein that accumulates in the nucleus is so small that it cannot be readily detected by conventional biochemical or immunological means. Here, we have used potentially more sensitive in vivo assays for nuclear access and action to ask whether the Notch intracellular domain does indeed gain access to the nucleus in response to ligand and whether it transduces Notch signals within the nucleus by regulating the transcription of downstream target genes.

Evidence for Ligand-Dependent Nuclear Access of the Notch Intracellular Domain

In the first series of experiments, we have used insertions of the Gal4-VP16 transcriptional activator at different sites within Notch protein to assay whether any portions of the receptor normally have access to the nucleus. The premise of this approach is that the Gal4-VP16 domain must gain access to the nucleus to activate transcription of a *UAS-lacZ* target gene and hence serves as an indicator for nuclear access of adjoining Notch sequences. We have found that Gal4-VP16 domains inserted in the intracellular domain, but not the extracellular domain, of Notch do indeed have access to the nucleus, as judged by their ability to activate *UAS-lacZ* transcription. Moreover, we show that access of a specific Gal4-VP16 insertion, GV3, positioned just carboxy-terminal to the Notch transmembrane domain is ligand-dependent and correlates with Notch signal–transducing activity. Thus, our results provide in vivo evidence that the intracellular domain of Notch gains access to the nucleus in response to ligand stimulation.

Evidence that Nuclear Access of the Intracellular Domain Is Necessary for Signal Transduction

We performed a second series of experiments to investigate whether nuclear access is required for Notch signal transduction. We first defined a minimal fragment of the Notch intracellular domain containing the CDC10 repeats that has intrinsic transducing activity (see also Roehl and Kimble, 1993) and then asked whether the intrinsic transducing activity associated with this fragment depends on its having access to the nucleus. We find that addition of sequences that permit or target this polypeptide to accumulate in the nucleus retain transducing activity, whereas sequences that target this polypeptide to membranes block the activity. Because the CDC10 repeats are essential for signal transduction by Notch (Greenwald and Seydoux, 1990; Kodoyianni et al., 1992; Lieber et al., 1993; Rebay et al., 1993), we interpret these results as evidence that the Notch intracellular domain must normally reach the nucleus to transduce Notch signals.

Evidence for a Direct Role of the Notch Intracellular Domain in Transcriptional Activation

We performed a third set of experiments to examine the role of the Notch intracellular domain in the nucleus and, specifically, whether it acts directly to regulate transcription. We inserted structural motifs that have well-defined roles in transcriptional activation and repression into the intracellular domain of intact Notch. We find that adding the VP16 activator domain renders the protein constitutively active, provided that it is inserted at a position which allows it to gain access to the nucleus irrespective of ligand. Moreover, the presence of the VP16 domain enhances the ability of Notch derivatives that carry the Gal4 DNA-binding domain to activate transcription of the *UAS-lacZ* target gene. In contrast, adding repressor motifs from either Engrailed or Hairy blocks the signal-transducing activity of the resulting Notch proteins as well as their ability to activate *UAS-lacZ* expression via the Gal4 DNA-binding domain. These results support the

hypothesis that the Notch intracellular domain directly transduces Notch signals within the nucleus by activating the transcription of downstream target genes.

Possible Mechanisms for Ligand-Dependent Nuclear Access of Notch

In principle, interactions between ligand and the extracellular domain of Notch might allow the intracellular domain to gain access to the nucleus by either of two mechanisms. In the first, these interactions would induce translocation of the entire receptor from the membrane to the nucleus. To be consistent with our findings, the translocated receptor would have to retain a membrane topology that would allow Gal4-VP16 domains inserted in the intracellular domain to bind and transcriptionally activate *UAS-lacZ* target genes while precluding the same activity by Gal4-VP16 domains inserted in the extracellular domain. In the second mechanism, which we favor, nuclear access would be afforded by one or more ligand-dependent proteolytic cleavages, which occur amino-terminal to the Notch intracellular domain and lead to its ability to move from the membrane to the nucleus.

A precedent for such a proteolytic processing mechanism is provided by the sterol-dependent nuclear import of DNA-binding domains of the Sterol Regulatory Element Binding Proteins 1 and 2 (SREBP-1 and SREBP-2) (reviewed in Brown and Goldstein, 1997). Each of the SREBPs is composed of three domains, an amino-terminal cytosolic domain that includes a transcription factor of the basic-helix-loop-helix class, a central domain consisting of two transmembrane domains flanking a short extracytosolic domain, and a carboxy-terminal cytosolic domain. A reduction in cholesterol abundance is sensed by the SREBP cleavage-activating protein (SCAP), which mediates a sequence-specific cleavage event in the extracytosolic domain. This first cleavage then precipitates a second cleavage, which occurs within the amino-terminal transmembrane domain. As a consequence of the second cleavage, the amino-terminal cytosolic domain is released from the membrane and translocates to the nucleus, where it binds cholesterol response genes and activates their transcription.

We suggest that the Notch intracellular domain may gain access to the nucleus by a similar mechanism to that of the SREBPs with ligand binding precipitating one or more cleavages, leading to the release of the intracellular domain from the membrane. Because the Gal4-VP16 domain inserted just downstream of the transmembrane domain of N^{+}-GV3 protein gains access to the nucleus in response to ligand, these cleavages would presumably occur in the extracellular or transmembrane domains, as in the case of SREBPs, or just carboxy-terminal to the transmembrane, within or amino-terminal to three basic amino acids that comprise a putative stop-transfer sequence.

The relationship of the proposed ligand-dependent cleavage(s) to other biochemically defined cleavages in Notch proteins is uncertain. When expressed in mammalian tissue culture cells, a truncated derivative of mouse Notch1 protein equivalent to *Drosophila* N^{ECN} protein, mNΔE, has constitutive activity. Moreover, this protein appears to be cleaved in a region close to the junction between the transmembrane and intracellular domains resulting in nuclear localization of at least some of the cleaved protein in at least some cells (Kopan et al., 1996). Although this cleavage has been assumed to occur within the intracellular domain (Kopan et al., 1996), the biochemical evidence is also compatible with a cleavage event within the transmembrane domain. Thus, it is possible that both the mNΔE and *Drosophila* N^{ECN} proteins are constitutively active because they mimic an intermediate normally generated during ligand-dependent processing of the extracellular domain of Notch and are further processed, like endogenous Notch protein, by cleavages that occur within or just carboxy-terminal to the transmembrane domain.

Notch proteins also appear to be cleaved at a second site in the extracellular domain as they mature and are exported to the cell surface to yield a disulfide bond–linked heterodimer composed of a large amino-terminal portion of the extracellular domain and the remainder of the protein (Blaumueller et al., 1997). This cleavage appears to depend on the activity of a membrane-associated metalloprotease, Kuzbanian, which is required for Notch signal transduction (Pan and Rubin, 1997). However, there is no evidence at present that Kuzbanian actually cleaves Notch or is involved in ligand-dependent processing of Notch.

We note that processing of β-amyloid precursor protein (β-APP) involves an intramembrane cleavage that depends on the function of Presenilin proteins (De-Strooper et al., 1998), which also appear to be required for normal Notch activity (reviewed in Greenwald, 1998). Presenilins may therefore have a common role in facilitating cleavages within or adjacent to the transmembrane domains of both Notch and β-APP proteins.

Previous genetic studies of Lin-12 and Notch have suggested that ligand activates Notch proteins by facilitating oligomerization (Greenwald and Seydoux, 1990; Heitzler and Simpson, 1993). If signal transduction by Notch depends on ligand-dependent cleavages within or amino-terminal to the transmembrane domain, it follows that oligomerization may serve to regulate the proteolytic activity responsible for this cleavage. For example, oligomerization may allow the recruitment of an extracellular or transmembrane protease. Alternatively, such a protease may always be associated with Notch, but it requires oligomerization to act.

Nuclear Access of Carboxy-Terminal Portions of the Notch Intracellular Domain in the Absence of Ligand

Our findings using N-GV4 proteins suggest that some carboxy-terminal fragments of the Notch intracellular domain gain access to the nucleus as a consequence of cleavages within the intracellular domain. However, in contrast to the ligand-dependent nuclear access we detect using N-GV3 proteins, none of the carboxy-terminal processing events suggested by our experiments with N-GV4 proteins appear to depend on ligand. Nor do they appear to correlate with Notch signal–transducing activity, except in proteins in which the resulting carboxy-terminal portions of the intracellular domain would

Table 1. Amino Acid Sequences at Joins in Deleted or Chimeric Proteins

Protein[a]	Joins	Amino Acid Sequence (/Linker/)[b]
N^{ECN}	**Notch/Notch**	**SVGCQN/id/TAAKHQ**
N^{Sev11}	Sev11/Notch	LVLVRK/**RAHGVT**
N^{EGF}	**Notch/Notch**	**CEIAVP/EDCTES**
N^+-GV1	**Notch/Gal4VP16**	**NGGSGS**/ppp/KLLSSI
	Gal4VP16/Notch	IDEYGG/**SGNDRY**
N^+-GV2	**Notch/Gal4VP16**	**EAAEFL**/gsppp/KLLSSI
	Gal4VP16/Notch	IDEYGG/s/**TAAKHQ**
N^+-GV1/2	**Notch/Gal4VP16**	**NGGSGS**/ppp/KLLSSI
	Gal4VP16/Notch	IDEYGG/s/**TAAKHQ**
N^+-GV3	**Notch/Gal4VP16**	**STORKR**/sgpp/KLLSSI
	Gal4VP16/Notch	IDEYGG/isgv/**RKRAHG**
N^+-GV4	**Notch/Gal4VP16**	**QAMIGS**/ppp/KLLSSI
	Gal4VP16/Notch	IDEYGG/**SPPPGQ**
N^+-G4	**Notch/Gal4**	**QAMIGS**/ppp/KLLSSI
	Gal4/Notch	TGLFVQ/**GSPPPG**
N^+-G4,en^{Rep}	**Notch/Gal4**	**QAMIGS**/ppp/KLLSSI
	Gal4/engrailed	QLTVSI/slaag/ALEDRC
	engrailed/**Notch**	PEKSAL/**GSPPPG**
N^+-G4,$PEST^-$	**Notch**	**QHNQQA**/s
N^+-G4,WRPW	**Notch/WRPW**	**SEAIYI**/qp/WRPW
N^+-G4,WGPS	**Notch/WGPS**	**SEAIYI**/qp/WGPS
N^{Sev11}-GV3	Sev11/Gal4VP16	LVLVRK/rrsgpp/KLLSSI
	Gal4VP16/**Notch**	IDEYGG/isgv/**RKRAHG**
N^{CDC10},MYR^+NLS^+	MYR^+/Flu	MGNKCCSKRQ/gtmagni/$[YPYDVPDYAG]_2$
	Flu/**Notch**	$[YPYDVPDYAG]_2$/sma/**PPAHQD**
	Notch/NLS^+	**QAMIGS**/PPKKKRKVED
MYR^- signal		MANKCCSKRQ
NLS^- signal		PPKTTRKVED
Sev11MYR^- signal	Sev11/MYR^-	LVLVRK/rrsagrt/MANKCCSKRQ

[a] As indicated in Figures 1 and 4 and in text. For the N^+-G4,$PEST^-$, N^+-G4,WRPW, and N^+-G4,WGPS proteins, the G4 insertion is the same as that shown for N^+-G4 protein and is composed of the first 99 amino acids of the Gal4 protein. For the N^+-G4,en^{Rep} protein, the G4 insertion is composed of the first 147 amino acids of the Gal4 protein (all of the GV insertions contain the same 147 amino acid domain fused at the carboxyl terminus of the VP16 activation [Sadowski et al., 1988]). For the N^{CDC10} series, all five proteins are identical to N^{CDC10},MYR^+NLS^+ except for the substitution of the mutated MYR or NLS signals, or the Sev11MYR^- signal. The Sev11 and N^{ECN} proteins are described, respectively, in Basler et al. (1991) and Rebay et al. (1993).

[b] Six amino acids of each protein adjacent to the join are shown (N sequences are in boldface), and the joins are listed in the amino-terminal to carboxyl-terminal order. The Flu, MYR, and NLS signals are shown in full. Linkers between the joins, when present, are indicated in lower case.

also contain insertions of the VP16 activating domain. At present, a minimalist view of these putative intracellular cleavages is that they reflect nonspecific degradative events that do not normally play a role in signal transduction, except perhaps in the potentially important process of down-regulating receptor activity. However, it remains possible that one or more of these processing events is essential for N signal–transducing activity once a ligand-dependent cleavage occurs upstream.

Signal Transduction by the Notch Intracellular Domain

In principle, the only limiting step in the mechanism of signal transduction by Notch may be the proposed ligand-dependent cleavage event that releases the intracellular domain from the membrane and allows it to enter the nucleus. In favor of this view, the Notch intracellular domain has intrinsic signal-transducing activity and is found predominantly in the nucleus when expressed on its own (Lieber et al., 1993; Struhl et al., 1993). Moreover, the intrinsic activity of a smaller domain composed primarily of the CDC10 repeats depends on its being allowed to enter the nucleus (Figure 3). However, Notch signal transduction also appears to depend on several proteins that associate physically with the Notch intracellular domain, such as Su(H), Dishevelled, Deltex, and Numb, as well as other proteins, such as Mastermind and Hairless (reviewed in Weinmaster, 1997; Greenwald, 1998). Hence, interactions between ligand and the Notch extracellular domain may be required not only to release the intracellular domain but also to initiate a series of other events within the cell that are required to generate a productive complex of this domain with other proteins.

What is the nature of this complex, and what is its role in the nucleus? At least in the embryonic ventral ectoderm, our findings support the view that the Notch intracellular domain functions in the nucleus to activate transcription of downstream target genes. Key targets for Notch-dependent transcriptional activation are genes of the *Enhancer of split* complex (*E[spl]*). These genes are required in the ventral ectoderm to specify epidermal versus neural differentiation (reviewed in Weinmaster, 1997; Greenwald, 1998). Morever, they have been shown to be direct targets for binding by the Su(H) protein (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995) and to be transcriptionally activated in response to Notch signaling (Jennings et al., 1994; Jarriault et al., 1995; Kopan et al., 1996; Eastman et al., 1997).

By analogy with EBVNA2, a viral coactivator protein that interacts with a mammalian Su(H) homolog CBF1 to convert it from a transcriptional repressor to a transcriptional activator (Hsieh and Hayward, 1995), the Notch intracellular domain, perhaps in association with other proteins, may combine with Su(H) to contribute an activation domain that allows Su(H) to activate the transcription of *E(spl)* genes as well as other target genes. We note that Su(H) is not required for all Notch-dependent developmental decisions (Lecourtois and Schweisguth, 1995; Wang et al., 1997), raising the possibility that Notch mediates some cell fate choices by associating with other DNA-binding transcription factors or by employing other mechanisms to transduce extracellular signals.

Experimental Procedures

Construction of Notch Transgenes

Wild-type and deleted derivatives of the *N* and *N-GV* coding sequences were inserted into a *Casper-hsp70* vector, which contains the *hsp70* promoter upstream of the insertion site and a 3′ UTR sequence from the *SV40* T-antigen gene downstream of the insertion site. The *N* coding sequence was derived from a *N* minigene (as in Struhl et al., 1993). The Gal4-VP16 coding sequence encodes the first 147 amino acids of Gal4, including the DNA-binding domain, and the carboxy-terminal 78 amino acids of VP16, including the activation domain (Sadowski et al., 1988). The Sev11 (Basler et al., 1991), Myristylation (Cross et al., 1984; Simon et al., 1985), NLS (Kalderon et al., 1984), and Flu epitope (Wilson et al., 1984) sequences have been described previously (Table 1). For some experiments, we also used a *pUAST* vector (Brand and Perrimon, 1993) to place *N* sequences under the control of a promoter that responds to Gal4. In these contructs, a >*y*+> flp-out cassette (Basler and Struhl, 1994) was introduced immediately upstream of the Notch sequences and excised after the constructs were inserted as stable transgenes in the *Drosophila* genome. The deduced amino acid sequences at the joins between Notch, Gal4-VP16, En, Hairy, and Sev11 proteins as well as between these sequences and the Myristylation, SV40 NLS, and Flu sequences in all of these constructs are listed in Table 1.

In Vivo Assays of Notch Transducing Activity, *UAS-lacZ* Expression, and Notch Expression

UAS-lacZ expression: embryos carrying one copy of the given *hsp70-N-GV* or *hsp70-N-G* transgene and one copy of the *UAS-lacZ* reporter (Fischer et al., 1988) were heat shocked for 1 hr at 37°C (or lower temperatures, as indicated), allowed to recover for 2 hr at 25°C, and then fixed and stained for β-gal expression by standard immunohistochemical methods (as in Struhl et al., 1993).

UAS-lacZ expression in Dl^{X43} versus Df^{43} *hsp70-Dl* embryos. Embryos derived from a cross between *hsp70-N-GV* Dl^{X43}/TM3, *ftz-lacZ* males and either *UAS-lacZ*; Df^{43}/*TM3, ftz-lacZ* or *UAS-lacZ*; Df^{43} *hsp70-Dl/TM3, ftz-lacZ* females were heat shocked and stained for β-gal expression as described above. Df^{43} mutant embryos were identified by the absence of striped *ftz-lacZ* expression. The *hsp70-Dl* transgene contains the coding sequence for Delta (Kopczynski et al., 1988) inserted in a *Carnegie20*-derived *hsp70* vector (Struhl et al., 1993).

Neuroblast segregation assay: embryos carrying a single copy of the given *hsp70-N* or *hsp70-N-GV* transgene were heat shocked for 30 min at 37°C, allowed to recover for 90 min at 25°C, and stained for Hunchback (Hb) expression, which marks newly segregated neuroblasts, by immunhistochemistry (as in Struhl et al., 1993). In experiments involving Notch derivatives that lack Gal4 or Gal4-VP16 domains, the Gal4/UAS system was also used to drive ubiquitous expression. For these experiments, embryos carrying a single copy of the given *UAS-N* transgene and either of two Gal4 driver genes, *arm-Gal4* (Sanson et al., 1996) or *Tubα1>Gal4-VP16*^{F442A} were stained for Hb expression. The *Tubα1>Gal4-VP16*^{F442A} transgene is composed of the *Tubα1* promoter, which is active in most or all cells (Basler and Struhl, 1994), positioned upstream of a *Gal4-VP16* coding sequence that contains the F442A mutation, which reduces the activity of the VP16 activation domain (Regier et al., 1993).

Epidermal rescue assay for N^- embryos: N^{kk11} arm^{YD35}/*FM7* females were crossed to males carrying a given *hsp70-N* or *hsp70-N-GV* transgene and embryos subjected to three 1 hr 37°C heat shocks interspersed with 3 hr recovery times at 25°C. Larval cuticles formed by the resulting embryos were assayed for the Armadillo segmentation phenotype (Lieber et al., 1993). For the various Notch derivatives that do not contain Gal4, the Gal4/UAS method was also used: for these experiments, N^{kk11} arm^{YD35} embryos carrying a given *UAS-N* gene and the *Tubα1>Gal4-VP16*^{F442A} transgene were derived from N^{kk11} arm^{YD35}/*FM7* females. In all cases, identical results were obtained using the *hsp70* promoter or the Gal4/UAS technique to express the same Notch protein.

Epidermal rescue assay for Dl^- embryos: Dl^{X43} hh^{10E}/*TM3* females were crossed to *hsp70-N-GV/+*; Df^{43} hh^{10E}/+ males, treated as above for N^{kk11} arm^{YD35} embryos, and the resulting cuticles scored for the Hedgehog segmentation phenotype. For all of the Notch derivatives that do not contain Gal4, equivalent experiments were also performed using the Gal4/UAS method (as described above for epidermal rescue of N^- embryos) and identical results obtained.

Assays for the subcellular distribution of Notch-derived proteins: a monoclonal antisera Mab179C6 (Fehon et al., 1991) was used to detect the intracellular domain of Notch. The epitope is present in the CDC10 bearing domain used in the experiments shown in Figure 3. Commerically available polyclonal anti-Gal4 (Santa Cruz) and monoclonal anti-Flu (BabCo) antisera were used to detect the Gal4 and Flu epitopes. For the N^{CDC10},*MYR NLS* series of transgenes, embryos carrying a *hsp70-flp* transgene, the *69B-Gal4* transgene (which drives the expression of *UAS*-target genes in most ectodermal cells; Brand and Perrimon, 1993), and a given *UAS*>y^+>N^{CDC10},*MYR NLS* transgene were heat shocked to remove the >y^+> Flp-out cassette, generating clones of cells that express the N^{CDC10},*MYR NLS* coding sequence.

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Detection of programmed cell death using fluorescence energy transfer.

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Rigel, Inc., 772 Lucerne Drive, Sunnyvale, CA 94086, USA.

Fluorescence energy transfer (FRET) can be generated when green fluorescent protein (GFP) and blue fluorescent protein (BFP) are covalently linked together by a short peptide. Cleavage of this linkage by protease completely eliminates FRET effect. Caspase-3 (CPP32) is an important cellular protease activated during programmed cell death. An 18 amino acid peptide containing CPP32 recognition sequence, DEVD, was used to link GFP and BFP together. CPP32 activation can be monitored by FRET assay during the apoptosis process.

PMID: 9518501 [PubMed - indexed for MEDLINE]

1: Curr Biol. 1998 Jun 18;8(13):771-4. Related Articles, Links

Indirect evidence for Delta-dependent intracellular processing of notch in Drosophila embryos.

Lecourtois M, Schweisguth F.

Ecole Normale Superiuer, CNRS ATIPE URA1857, Paris, France.

Cell-cell signaling mediated by the receptor Notch regulates the differentiation of a wide variety of cell types in invertebrate and vertebrate species, but the mechanism of signal transduction following receptor activation is unknown. A recent model proposes that ligand binding induces intracellular processing of Notch; the processed intracellular form of Notch then translocates to the nucleus and interacts with DNA-bound Suppressor of Hairless (Su(H)), a transcription factor required for target gene expression. As intracellular processing of endogenous Notch has so far escaped immunodetection, we devised a sensitive nuclear-activity assay to monitor indirectly the processing of an engineered Notch in vivo. First, we show that the intracellular domain of Notch, fused to the DNA-binding domain of Gal4, regulated transcription, in a delta-independent manner. Second, we show that full-length Notch, containing the Gal4 DNA-binding domain inserted 27 amino acids carboxy-terminal to the transmembrane domain, activated transcription in a delta-dependent manner. These results provide indirect evidence for a ligand-dependent intracellular processing event in vivo, supporting the view that Su(H)-dependent Notch signaling involves intracellular cleavage, and transcriptional regulation by processed Notch.

PMID: 9651681 [PubMed - indexed for MEDLINE]

1: Cell. 1985 Aug;42(1):93-104. Related Articles, Links

Entrez PubMed

Cell Press

The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family.

Maddon PJ, Littman DR, Godfrey M, Maddon DE, Chess L, Axel R.

PubMed Services

The surface glycoproteins T4 and T8 define different functional subsets of T lymphocytes and may act as recognition molecules mediating appropriate interactions between the T cell and its target. Previously we employed gene transfer and subtractive hybridization to isolate a T8 cDNA; now we have isolated and sequenced a cDNA clone encoding the T4 molecule. The deduced protein sequence reveals that T4 is an integral membrane protein that shares significant amino acid and structural homologies with members of the immunoglobulin supergene family. The overall structure of T4 consists of an N-terminal variable (V)-like domain, a joining (J)-like region, a third extracellular domain, a membrane-spanning region homologous to class II MHC beta-chains, and a highly charged cytoplasmic domain. Comparison of the protein sequences deduced from the T4 and T8 cDNAs reveals structural similarities consistent with their postulated role as recognition molecules, as well as differences suggesting that the two proteins recognize different structures on the target cell.

PMID: 2990730 [PubMed - indexed for MEDLINE]

Related Resources

1: Methods Cell Biol. 1994;43 Pt A:335-52. Related Articles, Links

Entrez PubMed

Regulation of protein activities by fusion to steroid binding domains.

Mattioni T, Louvion JF, Picard D.

Departement de Biologie Cellulaire, Universite de Geneve, Switzerland.

PubMed Services

Publication Types:
- Review
- Review, Tutorial

PMID: 7823870 [PubMed - indexed for MEDLINE]

Related Resources

NCBI PubMed National Library of Medicine NLM

PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM Books

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#31	Search **Cell** Field: **Journal,** Limits: **Publication Date from 1985 to 1985**	14:26:58	411
#30	Search **Madden** Field: **Author,** Limits: **Publication Date from 1985 to 1985**	14:26:14	40
#29	Search **Madden PJ** Field: **Author,** Limits: **Publication Date from 1985 to 1985**	14:25:40	0
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#25	Search **#23 AND #24** Field: **Pagination,** Limits: **Publication Date from 1998 to 1998**	14:22:01	1
#24	Search **771** Field: **Pagination,** Limits: **Publication Date from 1998 to 1998**	14:21:44	189
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#22	Search **Lecourtis** Field: **Author,** Limits: **Publication Date from 1998 to 1998**	14:20:50	0
#21	Search **Lecourtis M** Field: **Author,** Limits: **Publication Date from 1998 to 1998**	14:20:23	0
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#14	Search **Slices JJ** Field: **Author,** Limits: **Publication Date from 1998 to 1998**	14:05:07	0
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#5	Search **caspase detection** Field: **Title,** Limits: **Publication Date from 1980 to 1999**	13:49:25	6
#3	Search **#1 AND #2** Field: **Volume,** Limits: **Publication Date from 1999 to 1999**	13:30:07	2
#2	Search **96** Field: **Volume,** Limits: **Publication Date from 1999 to 1999**	13:29:48	4413
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☐ 1. Document ID: US 20030147810 A1

L3: Entry 1 of 23 File: PGPB Aug 7, 2003

PGPUB-DOCUMENT-NUMBER: 20030147810
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030147810 A1

TITLE: Compositions and methods for reporting of protease activity within the secretory pathway

PUBLICATION-DATE: August 7, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Ross, Brian D.	Ann Arbor	MI	US	
Rehemtulla, Alnawaz	Plymouth	MI	US	

US-CL-CURRENT: 424/9.6; 435/226, 435/252.3, 435/254.2, 435/317.1, 435/320.1, 435/325, 435/419, 435/6, 435/69.1, 536/23.2 , 800/8

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Image

☐ 2. Document ID: US 20030138843 A1

L3: Entry 2 of 23 File: PGPB Jul 24, 2003

PGPUB-DOCUMENT-NUMBER: 20030138843
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030138843 A1

TITLE: Method for determining and modifying protein/peptide solubility

PUBLICATION-DATE: July 24, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Waldo, Geoffrey S.	Espanola	NM	US	

US-CL-CURRENT: 435/7.1; 435/320.1, 435/325, 435/455, 435/69.7

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Image

☐ 3. Document ID: US 20030083467 A1

L3: Entry 3 of 23 File: PGPB May 1, 2003

PGPUB-DOCUMENT-NUMBER: 20030083467

PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030083467 A1

TITLE: Fusion proteins, DNA molecules, vectors, and host cells useful for measuring protease activity

PUBLICATION-DATE: May 1, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Germann, Ursula	Newton	MA	US	
Hoock, Thomas	Somerville	MA	US	
Kwong, Ann	Cambridge	MA	US	

US-CL-CURRENT: 530/350; 435/219, 435/23, 435/320.1, 435/325, 435/69.7, 536/23.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Image

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☐ 4. Document ID: US 20030064435 A1

L3: Entry 4 of 23 File: PGPB Apr 3, 2003

PGPUB-DOCUMENT-NUMBER: 20030064435
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030064435 A1

TITLE: Compositions and methods for protein secretion

PUBLICATION-DATE: April 3, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Weiner, Joel Hirsch	Edmonton		CA	
Turner, Raymond Joseph	Calgary		CA	

US-CL-CURRENT: 435/69.1; 435/320.1, 435/325, 530/350, 536/23.5

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 5. Document ID: US 20020132327 A1

L3: Entry 5 of 23 File: PGPB Sep 19, 2002

PGPUB-DOCUMENT-NUMBER: 20020132327
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020132327 A1

TITLE: METHOD FOR IDENTIFYING PROTEASES, PROTEASE TARGET SITES AND REGULATORS OF PROTEASE ACTIVITY IN CELLS

PUBLICATION-DATE: September 19, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
HAY, BRUCE A.	PASADENA	CA	US	
HAWKINS, CHRISTINE V.	PARK ORCHARDS		AU	

US-CL-CURRENT: 435/195; 435/183, 435/243, 435/325, 435/4, 435/410, 435/7.1, 530/350, 536/23.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 6. Document ID: US 20020016296 A1

L3: Entry 6 of 23 File: PGPB Feb 7, 2002

PGPUB-DOCUMENT-NUMBER: 20020016296
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020016296 A1

TITLE: Aspartylprotease

PUBLICATION-DATE: February 7, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Haass, Christian	Icking		DE	
Steiner, Harald	Muenchen		DE	
Fechteler, Katja	Wiesbaden		DE	
Kostka, Marcus	Mainz		DE	

US-CL-CURRENT: 514/16; 530/329

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 7. Document ID: US 6610906 B1

L3: Entry 7 of 23 File: USPT Aug 26, 2003

US-PAT-NO: 6610906
DOCUMENT-IDENTIFIER: US 6610906 B1

TITLE: Nucleotide sequences for gene regulation and methods of use thereof

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 8. Document ID: US 6528276 B1

L3: Entry 8 of 23 File: USPT Mar 4, 2003

US-PAT-NO: 6528276
DOCUMENT-IDENTIFIER: US 6528276 B1

TITLE: Fusion proteins, DNA molecules, vectors, and host cells useful for measuring protease activity

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 9. Document ID: US 6448087 B1

L3: Entry 9 of 23 File: USPT Sep 10, 2002

US-PAT-NO: 6448087
DOCUMENT-IDENTIFIER: US 6448087 B1

TITLE: Method for determining and modifying protein/peptide solubility

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 10. Document ID: US 6383745 B1

L3: Entry 10 of 23 File: USPT May 7, 2002

US-PAT-NO: 6383745
DOCUMENT-IDENTIFIER: US 6383745 B1

TITLE: Methods of screening for anti-microbial utilizing aarC and compositions thereof

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 11. Document ID: US 6335178 B1

L3: Entry 11 of 23 File: USPT Jan 1, 2002

US-PAT-NO: 6335178
DOCUMENT-IDENTIFIER: US 6335178 B1

TITLE: Compositions and methods for protein secretion

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 12. Document ID: US 6333167 B1

L3: Entry 12 of 23 File: USPT Dec 25, 2001

US-PAT-NO: 6333167
DOCUMENT-IDENTIFIER: US 6333167 B1

TITLE: Methods and reagents for identifying inhibitors of proteolysis of membrane-associated proteins

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 13. Document ID: US 6303317 B1

L3: Entry 13 of 23 File: USPT Oct 16, 2001

US-PAT-NO: 6303317
DOCUMENT-IDENTIFIER: US 6303317 B1
**** See image for Certificate of Correction ****

TITLE: Peptide probes and methods for making the same

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 14. Document ID: US 6117639 A

L3: Entry 14 of 23 File: USPT Sep 12, 2000

US-PAT-NO: 6117639
DOCUMENT-IDENTIFIER: US 6117639 A

TITLE: Fusion proteins, DNA molecules, vectors, and host cells useful for measuring protease activity

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 15. Document ID: US 6022952 A

L3: Entry 15 of 23 File: USPT Feb 8, 2000

US-PAT-NO: 6022952
DOCUMENT-IDENTIFIER: US 6022952 A

TITLE: Compositions and methods for protein secretion

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 16. Document ID: US 5858367 A

L3: Entry 16 of 23 File: USPT Jan 12, 1999

US-PAT-NO: 5858367
DOCUMENT-IDENTIFIER: US 5858367 A

TITLE: Methods for screening for antimicrobials utilizing AarC and compositions thereof

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 17. Document ID: US 5650389 A

L3: Entry 17 of 23 File: USPT Jul 22, 1997

US-PAT-NO: 5650389
DOCUMENT-IDENTIFIER: US 5650389 A

TITLE: Methods for the inhibition of complement activation

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 18. Document ID: WO 9947640 A1

L3: Entry 18 of 23 File: EPAB Sep 23, 1999

PUB-NO: WO009947640A1
DOCUMENT-IDENTIFIER: WO 9947640 A1
TITLE: METHOD FOR IDENTIFYING PROTEASES, PROTEASE TARGET SITES AND REGULATORS OF PROTEASE ACTIVITY IN CELLS

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 19. Document ID: US 6528276 B1

L3: Entry 19 of 23 File: DWPI Mar 4, 2003

DERWENT-ACC-NO: 2003-370632
DERWENT-WEEK: 200335

TITLE: Assaying protease activity in a cell, useful in diagnosing and treating e.g. Alzheimer's disease and hypertension, comprises using a fusion protein with a protease cleavage site and a ligand- and DNA-binding domain, and a reporter gene

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 20. Document ID: JP 2002543797 W DE 19920514 A1 WO 200068416 A2 EP 1177313 A2

L3: Entry 20 of 23 File: DWPI Dec 24, 2002

DERWENT-ACC-NO: 2001-000492
DERWENT-WEEK: 200313

TITLE: Identifying protease that cleaves membrane-associated substrate, useful e.g. for developing specific therapeutic inhibitors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 21. Document ID: WO 200066615 A1 AU 200049804 A

L3: Entry 21 of 23 File: DWPI Nov 9, 2000

DERWENT-ACC-NO: 2001-007205
DERWENT-WEEK: 200101

TITLE: Novel chimeric protease detector protein comprising repressor domain, protease cleavage domain that is specific for protease to be assayed and reporter domain, useful to detect presence or amount of active protease

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Draw Desc | Image

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☐ 22. Document ID: WO 200012727 A1 AU 9960234 A US 6117639 A EP 1109920 A1 JP 2002523102 W US 20030083467 A1 AU 760441 B

L3: Entry 22 of 23 File: DWPI Mar 9, 2000

DERWENT-ACC-NO: 2000-246756
DERWENT-WEEK: 200337

TITLE: Novel fusion protein comprising a protease cleavage site, a ligand binding domain, and a DNA binding domain useful for characterizing proteases, detecting viral infection, and screening for protease inhibitors

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Drawn Desc | Clip Img | Image

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☐ 23. Document ID: WO 9947640 A1 US 20020132327 A1

L3: Entry 23 of 23 File: DWPI Sep 23, 1999

DERWENT-ACC-NO: 1999-571832
DERWENT-WEEK: 200270

TITLE: A protease-dependent reporter fusion protein construct useful in assays to identify proteases

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments KWIC | Drawn Desc | Image

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Terms	Documents
L2 same (protease cleavage)	23

Display Format: - Change Format

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L9 ANSWER 1587 OF 1591 USPATFULL on STN

ACCESSION NUMBER:	94:73218 USPATFULL
TITLE:	Method of preparing a B-1,4 glycan matrix containing a bound fusion protein
INVENTOR(S):	Kilburn, Douglas G., Vancouver, Canada Miller, Robert C., North Vancouver, Canada Gilkes, Neil, Vancouver, Canada Warren, R. Antony J., Vancouver, Canada
PATENT ASSIGNEE(S):	University of British Columbia, Vancouver, Canada (non-U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5340731		19940823
APPLICATION INFO.:	US 1992-865095		19920408 (7)

RELATED APPLN. INFO.: Continuation-in-part of Ser. No. US 1990-603987, filed on 25 Oct 1990, now patented, Pat. No. US 5202247 which is a division of Ser. No. US 1988-216794, filed on 8 Jul 1988, now patented, Pat. No. US 5137819

DOCUMENT TYPE:	Utility
FILE SEGMENT:	Granted
PRIMARY EXAMINER:	Naff, David M.
LEGAL REPRESENTATIVE:	Rae-Venter, Barbara
NUMBER OF CLAIMS:	19
EXEMPLARY CLAIM:	1
NUMBER OF DRAWINGS:	35 Drawing Figure(s); 20 Drawing Page(s)
LINE COUNT:	1617

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A fusion protein that can function as a removable label is prepared containing a polypetide such as an enzyme and an amino acid sequence having a substrate binding region of a polysaccharidase such as cellulase that has essentially no polysaccharidase activity. By contacting the fusion protein with a .beta.-1,4 glycan matrix such as cellulose, the substrate binding region binds to the matrix to immobilize the polypeptide. The polypetide or fusion protein can be removed from the matrix with a protease capable of cleaving a specific **protease cleavage** site, or with a solution having a low ionic strength or a high pH. The fusion protein can be prepared by recombinant DNA technology.

L4 ANSWER 191 OF 195 BIOTECHNO COPYRIGHT 2003 Elsevier Science B.V. on STN
ACCESSION NUMBER: 1998:28445426 BIOTECHNO
TITLE: Use of the yeast three-**hybrid** system as a tool to study **caspases**
AUTHOR: Van Criekinge W.; Van Gurp M.; Decoster E.; Schotte P.; Van de Craen M.; Fiers W.; Vandenabeele P.; Beyaert R.
CORPORATE SOURCE: R. Beyaert, Department of Molecular Biology, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium. E-mail: rudi.beyaert@lmb1.rug.ac.be
SOURCE: Analytical Biochemistry, (01 OCT 1998), 263/1 (62-66), 19 reference(s)
CODEN: ANBCA2 ISSN: 0003-2697
DOCUMENT TYPE: Journal; Article
COUNTRY: United States
LANGUAGE: English
SUMMARY LANGUAGE: English

AB **Caspases** are a family of heteromeric (p20/p10) cysteine **proteases** with important functions in the regulation of apoptosis and inflammation. Up to now, tools to **identify** new substrates for **caspases** have mostly been limited to the random screening of in vitro translated proteins that are known, or assumed, to play a role in apoptosis. We describe the use of a yeast three- **hybrid** approach as a tool that adapts the classical two-**hybrid** system to the needs of heteromeric **caspases** for functional dissection of known interactions or screening for physiological substrates and inhibitors. Functional heteromeric **caspase**-1 was obtained by coexpression of p20(Cys285Ser) and p10 **caspase**-1 subunits that were each fused to the Gal4 DNA-binding domain. Upon coexpression of a third **hybrid** of the Gal4 activation domain and the viral **caspase**-1 pseudosubstrate inhibitors CrmA or p35, or the prototype physiological **caspase**-1 substrate prointerleukin-1.beta., a functional Gal4 transcription factor could be reconstituted. In contrast, no interaction was found between CrmA or p35 and the immature p45 or p30 precursor forms of **caspase**-1. Therefore, the three-**hybrid** system might allow screening for new physiological substrates and inhibitors of heteromeric **caspases**.

L4 ANSWER 193 OF 195 BIOTECHNO COPYRIGHT 2003 Elsevier Science B.V. on STN
ACCESSION NUMBER: 1998:28133943 BIOTECHNO
TITLE: An induced proximity model for **caspase**-8 activation
AUTHOR: Muzio M.; Stockwell B.R.; Stennicke H.R.; Salvesen G.S.; Dixit V.M.
CORPORATE SOURCE: V.M. Dixit, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, United States.
E-mail: dixit@gene.com
SOURCE: Journal of Biological Chemistry, (30 JAN 1998), 273/5 (2926-2930), 23 reference(s)
CODEN: JBCHA3 ISSN: 0021-9258
DOCUMENT TYPE: Journal; Article
COUNTRY: United States
LANGUAGE: English
SUMMARY LANGUAGE: English

AB The assembly of the CD-95 (Fas/Apo-1) receptor death-inducing signaling complex occurs in a hierarchical manner; the death domain of CD-95 binds to the corresponding domain in the adapter molecule Fas-associated death domain (FADD) Mort-1, which in turn recruits the zymogen form of the death **protease caspase**-8 (FLICE/Mach-1) by a homophilic interaction involving the death effector domains. Immediately after recruitment, the single polypeptide FLICE zymogen is proteolytically processed to the active dimeric species composed of large and small catalytic subunits. Since all **caspases** cleave their substrates after Asp residues and are themselves processed from the single- chain zymogen to the two-chain active enzyme by cleavage at internal Asp residues, it follows that an upstream **caspase** can process a downstream zymogen. However, since FLICE represents the most apical **caspase** in the Fas pathway, its mode of activation has been enigmatic. We hypothesized that the FLICE zymogen possesses intrinsic enzymatic activity such that when approximated, it autoprocesses to the active **protease**. Support for this was provided by (i) the synthesis of **chimeric** F(pk)3FLICE molecules that can be oligomerized in vivo by the synthetic cell-permeable dimerizer FK1012H2. Cells transfected with F(pk)3FLICE underwent apoptosis after exposure to FK1012H2; (ii) the creation of a nonprocessable zymogen form of FLICE that retained low but **detectable protease** activity.

PALM INTRANET

Day : Thursday
Date: 8/28/2003
Time: 08:38:52

Inventor Name Search Result

Your Search was:

Last Name = LAM
First Name = ERIC

Application#	Patent#	Status	Date Filed	Title	Inventor Name
90006417	Not Issued	414	10/22/2002	METHOD FOR CUTTING UNHARDENED CONCRETE WITH A SOFT CONCRETE SAW	LAMORTE, ERIC
90006320	Not Issued	414	07/09/2002	RAIN DRAINAGE GROOVES IN A ROAD AND APPARATUS FOR MAKING THEM	LAMORTE, ERIC
90006081	Not Issued	424	08/13/2001	LIPOIC ACID IN TOPICAL COMPOSITIONS	LAMORTE, ERIC A.
60456936	Not Issued	020	03/25/2003	POLYMETAL-BINDING PROTEINS COMPOSITION AND USES THEREOF	LAMIOT, ERIC
60432027	Not Issued	020	12/10/2002	FOOD COMPOSITION AND USES THEREOF	LAMIOT, ERIC
60343677	Not Issued	020	12/28/2001	METHOD FOR OPTIMIZING A PROCESSING TIME FOR A SEMICONDUCTOR MANUFACTURING PROCEDURE	LAMA, ERIC
60269945	Not Issued	020	02/19/2001	METHOD FOR SHARING IMAGE REVENUES AND APPARATUS FOR PERFORMING THE SAME	LAMBRECHT, ERIC
60269910	Not Issued	020	02/19/2001	METHOD FOR PRESENTING IMAGES AND APPARATUS FOR PERFORMING THE SAME	LAMBRECHT, ERIC
60227041	Not Issued	020	08/23/2000	RIM SHIELD	LAMBACK, ERIC
60158701	Not Issued	159	10/08/1999	SYSTEM FOR DETECTING SPECIFIC DNA SEQUENCES USING DNA BINDING SITES	LAM , ERIC
60153146	Not	159	09/09/1999	SYSTEM FOR DETECTING	LAM , ERIC

	Issued			SPECIFIC DNA SEQUENCES USING DNA BINDING SITES	
60138068	Not Issued	159	06/08/1999	COMPOSITIONS AND METHODS FOR TARGETED GENE INSERTION	LAM , ERIC
60132358	Not Issued	159	05/04/1999	COMPOSITIONS AND METHODS FOR DETECTION OF ACTIVE PROTEASES	LAM , ERIC
60096623	Not Issued	159	08/14/1998	POINT-OF-SALE SYSTEM ADAPTED FOR POSTAL ENVIRONMENTS	LAM , ERIC
60083541	Not Issued	159	04/29/1998	GENE REQUIRED FOR GONADOGENESIS IN CAENORHABDITIS ELEGANS, MUTANT ALLELES OF THIS GENE AND METHODS OF IDENTIFYING ENHANCERS AND SUPPRESSORS OF THIS GENE	LAMBIE , ERIC J.
60069447	Not Issued	159	12/15/1997	GENE REQUIRED FOR GONADOGENESIS IN CAENORHABDITIS ELEGANS, MUTANT ALLELES OF THIS GENE AND METHODS OF IDENTIFYING ENHANCERS AND SUPPRESSORS OF THIS GENE	LAMBIE , ERIC J.
10222761	Not Issued	020	08/15/2002	METHOD AND SYSTEM FOR DELIVERING MULTIPLE SERVICES ELECTRONICALLY TO CUSTOMERS VIA A CENTRALIZED PORTAL ARCHITECTURE	LAM, ERIC CHEUKFUNG
10099410	6608162	150	03/15/2002	SPRAY-DRIED PHENOL FORMALDEHYDE RESINS	LAM, ERIC KENNETH
10080287	Not Issued	019	02/19/2002	METHOD FOR SHARING IMAGE REVENUES AND APPARATUS FOR PERFORMING THE SAME	LAMBRECHT, ERIC
10079943	Not	019	02/19/2002	METHOD FOR	LAMBRECHT, ERIC

	Issued			PRESENTING IMAGES AND APPARATUS FOR PERFORMING THE SAME	
10009472	Not Issued	071	03/29/2002	COMPOSITIONS AND METHODS FOR DETECTION OF ACTIVE PROTEASES	LAM, ERIC
10009054	Not Issued	030	04/29/2002	COMPOSITIONS AND METHODS FOR TARGETED GENE INSERTION	LAM, ERIC
09948156	Not Issued	019	09/07/2001	RIM SHIELD	LAMBACK, ERIC
09757070	Not Issued	030	01/08/2001	METHOD AND SYSTEM FOR FACILITATING PARTS PROCUREMENT AND PRODUCTION PLANNING ACROSS AN EXTENDED SUPPLY CHAIN	LAMBERT, ERIC
09752090	Not Issued	041	12/29/2000	METHOD AND SYSTEM FOR PROVIDING AN END-TO-END BUSINESS PROCESS FOR ELECTRONIC SUPPLIER QUALIFICATION AND QUALITY MANAGEMENT	LAMBERT, ERIC T.
09751585	Not Issued	041	12/29/2000	METHOD AND SYSTEM FOR ELECTRONICALLY QUALIFYING SUPPLIER PARTS	LAMBERT, ERIC T.
09658031	Not Issued	020	09/08/2000	SYSTEM FOR DETECTING SPECIFIC DNA SEQUENCES USING DNA BINDING SITES	LAM, ERIC
09247719	6618477	150	02/09/1999	SYSTEM AND METHOD FOR NON-INTRUSIVELY DISPLAYING INFORMATION TO A USER IN A TELECOMMUNICATIONS SYSTEM	LAMBIASE , ERIC
09204876	Not Issued	161	12/03/1998	GENE REQUIRED FOR GONADOGENSIS IN CAENORHABDITIS ELEGANS, MUTANT ALLELES OF THIS GENE AND METHODS OF IDENTIFYING	LAMBIE , ERIC J.

				ENHANCERS AND SUPPRESSORS OF THIS GENE	
08931293	5938072	150	09/16/1997	ROLLED COIN DISPENSER	LAMOUREUX , ERIC
08454670	Not Issued	161	05/31/1995	PORTABLE FIRST AID APPLIANCE FOR WASHING AN EYE AND/OR THE SKIN	LAMI , ERIC
08442360	Not Issued	163	05/16/1995	PAINTBALL HAVING IMPROVED AERODYNAMIC PERFORMANCE	LAMORTE , ERIC A.
08375778	5629470	150	01/20/1995	TRANSGENIC PLANTS AND PLANT CELLS WITH ENHANCED PATHOGEN RESISTANCE AND RELATED METHODS	LAM , ERIC
08108306	Not Issued	161	08/19/1993	TRANSPARENT AUTOMOTIVE SECURITY SYSTEM FOR STEERING COLUMN	LAM , ERIC
07752480	Not Issued	161	11/01/1991	THERMOMETER	LAMONT-GREGORY , ERIC
07681611	5191323	150	03/25/1991	REMOTE POWER ON CONTROL DEVICE	LAMBOLEY , ERIC
07649521	5223419	150	02/01/1991	ALTERATION OF GENE EXPRESSION IN PLANTS	LAM , ERIC
07435105	Not Issued	166	11/13/1989	REMOTE POWER ON CONTROL DEVICE	LAMBOLEY , ERIC
07323533	4990607	150	03/14/1989	ALTERATION OF GENE EXPRESSION IN PLANTS	LAM , ERIC
07272169	5023179	150	11/14/1988	PROMOTER ENHANCER ELEMENT FOR GENE EXPRESSION IN PLANT ROOTS	LAM , ERIC
07076816	4815101	150	07/23/1987	STEP-BY-STEP REMOTE LOCATING SYSTEM FOR REPEATERS IN A PCM LINK	LAMAIGNERE , ERIC F.H.
07037267	4850915	150	04/10/1987	APPARATUS FOR DEPLOYING AND SUPPORTING A LARGE APERTURE VOLUMETRIC ARRAY IN A MEDIUM	LAMBERT , ERIC M.
06852908	Not Issued	164	04/16/1986	VIDEO DISPLAY UNIT OR SIMILAR ARTICLE	LAMBERTON , ERIC W.

06496335	4518160	150	05/23/1983	FLAT ARTICLE STACKING AND TRAY LOADING APPARATUS	LAMBRECHTS , ERIC G.
06437402	Not Issued	164	10/28/1982	SONOBUOY OF DEPLOYING HYDROPHONES	LAMBERT , ERIC M.
06209236	Not Issued	166	11/21/1980	FLAT ARTICLE STACKING AND TRAY LOADING APPARATUS	LAMBRECHTS , ERIC G. Y.

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60428336	Not Issued	020	11/22/2002	USE OF VIRUS-INDUCED GENE SILENCING FOR IDENTIFICATION OF PLANT GENES HAVING A ROLE IN PLANT PATHOGEN RESISTANCE	DEL POZO, OLGA
60132358	Not Issued	159	05/04/1999	COMPOSITIONS AND METHODS FOR DETECTION OF ACTIVE PROTEASES	DEL POZO , OLGA

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